The Role of Orexin-A in Anxiety and the Emission of Ultrasonic Vocalizations in Rats

Alexa Morden

Department of Psychology

Submitted in partial fulfillment
of the requirements for the degree of

MA Psychology

Faculty of Graduate Studies, Brock University
St. Catharines, Ontario

List of Abbreviations

5-HT_1A- 5-hydroxytryptamine (serotonin) receptor 1A

ACTH- Adrenocorticotropic Hormone

AHA- Anterior Hypothalamic Area

ANOVA- Analysis of Variance

CCh- Carbachol

CRF- Corticotropin Releasing Factor

dB- Decibels

DSM- Diagnostic and Statistical Manual of Mental Disorders

GABA- Gamma-Amino Butyric Acid

HPA- Hypothalamic-Pituitary-Adrenal Axis

kHz- Kilohertz

LDT- Laterodorsal Tegemental Nucleus

LSD- Least Squares Difference

mCCP- meta-Chlorophenylpiperazine

MPOA- Medial Preoptic Area

mRNA- messenger RNA

ORX- Orexin- A

OUT- Outside of Medial Preoptic and Anterior Hypothalamic Areas

OX1R- Orexin Type 1 Receptor

OX2R- Orexin Type 2 Receptor

PVN- Paraventricular Nucleus of the Hypothalamus

SAL- Saline

SSRI- Selective Serotonin Reuptake Inhibitor

USV- Ultrasonic Vocalization

Abstract

Central administration of orexin-A has been shown to activate autonomic arousal in rats, reliably inducing anxiety-like behaviours in the open field. To date, there has yet to be a study investigating the role of orexin-A in the communication of such negative affective state. In the current study, forty-six adult male rats were chronically cannulated and administered orexin-A into the medial preoptic area/anterior hypothalamic area to determine the effect of this neuropeptide on anxiety-like behaviour and the production of 22 kHz aversive ultrasonic vocalizations. It was found that intracerebral administration of orexin-A increased autonomic arousal as measured by a significant increase in fecal boli output, however orexin-A did not significantly affect locomotor activity or induce 22 kHz calling. These data suggest that orexin-A is involved in the regulation of the autonomic aspect of anxiety-like behaviour but not in the vocal communication of such negative affect.

Keywords: Orexin-A; Anxiety-like behaviour; 22 kHz calls; Ultrasonic vocalizations; Rat

Acknowledgements

The author's research is supported by an operating grant from the Natural Sciences and Engineering Research Council of Canada. The author is grateful to Dr. Stefan Brudzynski, Dr. Dawn Good, Dr. Cheryl McCormick, Mike Silkstone, Feather Nixon, Sean Robb, and SJR.

Table of Contents

List of Tables

List of Figures

The Role of Orexin-A in Anxiety and the Emission of Ultrasonic Vocalizations in Rats

Anxiety disorders are the most commonly diagnosed mental health issues in the United States and Canada (Barlow, 2002; Health Canada, 2009), and represent the second leading cause of visits to a physician after routine physical exams (Barlow, 2002). While fear is understood as an innate primary emotion that serves to alert one to a tangible threat and arouse the body for action, anxiety is conceptualized as a fixation on possible negative events in the future, leading to hypervigilance of one's environment and apprehension (Barlow, 2012; 5th ed; *Diagnostic and Statistical Manual of Mental Disorders*, American Psychiatric Association, 2013). Panic, an extreme and unexpected form of fear (Freeman & Freeman, 2012), is characterized by episodes of autonomic arousal accompanied by psychological ideations (such as a fear of losing control or imminent death) peaking within 10 minutes after onset (DSM- 5, American Psychiatric Association, 2013). Many who seek medical attention for unexplained medical issues such as heart palpitations, faintness, bowel upset, vertigo and dizziness often subsequently meet the criteria for an anxiety disorder (Barlow, 2002). Typified by excessive or persistent fear or anxiety, these disorders include separation anxiety, selective mutism, specific phobia, social anxiety disorder, panic disorder, agoraphobia, generalized anxiety disorder, substance/medication induced anxiety disorder, anxiety disorders due to another medical condition, and other specified anxiety disorder (DSM-5, 2013). Once diagnosed, the symptoms of anxiety disorders are often chronic and recurrent for many individuals despite intervention (Barlow, 2002). As such, it has been estimated that anxiety disorders cost approximately 42.3 billion dollars per year

(Greenberg et al., 1999) largely due to the use of health care services and loss of productivity and absenteeism in the workplace (Barlow, 2002).

Many theories have been put forth in an attempt to explain the etiology of anxiety and its disorders, whether psychoanalytic, behavioural, cognitive or neurobiological (Freeman & Freeman, 2012). Seminal work by Donald Klein in 1959 was able to dissociate panic and anxiety. He was also able to show that sodium lactate and carbon dioxide could induce the panic, leading to the conclusion that such behaviours could result from the brain's perceived lack of oxygen, or a feeling of suffocation (Freeman & Freeman, 2012). David Clark and Hans Eysenck expanded on this idea theorizing that some individuals demonstrate high baseline levels of cortical arousal matched with a propensity for extreme autonomic reactivity (Barlow, 2012), and experience panic due to catastrophically misinterpreting physiological arousal of the body as dangerous and potentially fatal (Freeman & Freeman, 2012).

Jeffrey Gray has speculated that the neural basis of such behaviours compose a *'behavioural inhibition system'* regulated by the septal area, hippocampus, and structures of the Papez circuit, and contributing to the individual reacting to new situations and/or punishment with extreme behavioural inhibition and decreased behavioural approach (Barlow, 2012). Fear and panic on the other hand, are regulated by an alternate system, the *'fight or flight system*', which initiates defensive aggression or escape behaviours in aversive situations and is mediated by the periaqueductal gray and medial hypothalamic control of the amygdala (Barlow, 2012). The theory of behavioural inhibition is

supported by research with human subjects which has shown that individuals presenting with behavioural inhibition show hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis (Tykra et al., 2006).

Animal models of anxiety vulnerability are often used to study individual differences in trait anxiety. The Wistar Kyoto rat is a strain shown to demonstrate behavioural inhibition and hypervigilance, traits that are suggested by the hypervigilance-avoidance theory to play a key role in the development of anxiety disorders (McAuley, Stewart, Webber, Cromwell, Servatius, & Pang, 2009). The Otsuka Long Evans Tokushima Fatty rat, which lacks the cholecystokinin type 1 receptor (Schroeder & Weller, 2010), and wild type rats selectively bred for high anxiety-related behaviour in the elevated plus maze (Roy, Chapillon, Jeljeli, Caston & Belzung, 2009) are also used to study these effects.

Research with such animals and human subjects has shown that anxiety can be induced in some individuals through protocols that manipulate respiration (through exercise, hyperventilation, or CO^2 inhalation) or by increasing autonomic arousal [through administration of epinephrine, isoproterenol (stimulating beta-andrenergic receptors), yohimbine (an alpha-andrenergic antagonist), caffeine, cholecystokinin, benzodiazepine antagonists, mCCP (serotonin agonist), lactate, and acetylcholine] (Barlow, 2012). Currently two classes of drugs have been shown to have clinical efficacy in ameliorating these anxiogenic effects, benzodiazepines and selective serotonin reuptake inhibitors (Julien, Advokat & Comaty, 2008).

Treatments for anxiety and its disorders target transmitter systems in the brain known to regulate structures of the limbic system (Julien et al., 2008). Benzodiazepines are a group of drugs used for the short term relief of symptoms of anxiety exerting their anxiolytic action by facilitating the binding of GABA to $GABA_A$ receptors, thus increasing inhibitory tone on structures including the amygdala, orbitofrontal cortex and insula. However, given the risk of dependency with the use of benzodiazepines, serotonin receptor antagonists are more often used in the treatment of anxiety disorders. Selective serotonin reuptake inhibitor (SSRI) antidepressants, 5-HT $_{1A}$ agonists in particular, decrease neuronal activity in structures known to regulate anxiety-like behaviours (Julien et al., 2008). Recent research has shown that a group of neuropeptides known as orexins, or hypocretins, are involved in the regulation of anxiety-like behaviours and offer a new and exciting opportunity for anxiety research (Suzuki, Beuckmann, Shikata, Ogura, & Sawai, 2005).

Two endogenous neuropeptides, orexin-A and orexin-B, and their two receptor types, OX_1 receptor (OX_1R) and OX_2 receptor (OX_2R), were identified by Sakurai and colleagues in 1998 (Sakurai et al., 1998). Orexins were named after the Greek word for appetite (orexis), due to the fact that the mRNA precursor prepro-orexin is found primarily in the lateral hypothalamus which is known to regulate consummatory behaviour (Sakurai et al., 1998). However, in the same year, this group of hypothalamic neuropeptides were discovered by De Lecea and colleagues and named hypocretins due to their similar amino acid structure to the incretin group of hormones (De Lecea, et al.,

1998). It is currently estimated that orexin neurons constitute approximately 1,800 neurons within the lateral hypothalamus (Henny & Jones, 2006).

Direct innervations to orexin neurons arise from all regions of the cortex with the exception of the cerebellum (Yoshida et al., 2006). Primary projections to orexinergic neurons originate in the basal forebrain, limbic structures including the amygdala, and the brainstem, including the periaqueductal gray and dorsal raphe nucleus. The highest density of afferent connections is found in the lateral septum, the bed nucleus of the stria terminalis (BNST), the medial preoptic area (MPOA), and the posterior hypothalamus (Yoshia et al., 2006).

OX_1R exclusively binds orexin-A, while OX_2R is non-selective for orexin-A and orexin-B (Sakurai et al., 1998). OX_1R and OX_2R are found throughout the hypothalamus, although OX_2R receptor expression is most dense in the regions of the paraventricular nucleus (CRF neurons), lateral hypothalamic area (feeding), and the tuberomammillary nucleus (histamine), as well as the ventral tegmental area (dopamine) and raphe nuclei (serotonin) (Marcus et al., 2001). OX_1R mRNA has been identified primarily in the prelimbic and infralimbic cortices as well as the ventromedial region of the hypothalamus, the hippocampal formation, and the raphe nucleus, and found most densely in the locus coeruleus, noradrenergic cells, and cholinergic cells of the LDT (Marcus et al., 2001). mRNA for OX_2R have been localized to the paraventricular nucleus of the hypothalamus, the nucleus accumbens, and cerebral cortex (Trivedi et al.,

1998), and most abundantly in the deep layers of the cortex and areas of the basal forebrain including the septal nucleus and diagonal band, whose serotonergic and cholinergic neurons are stimulated by orexin (Marcus et al., 2001).

Given the vast connections of orexin neurons throughout the brain, Yoshida et al. (2006) have suggested that these neurons are involved in the integration of various sensory and motor abilities for the promotion of behavioural arousal in response to disruptions to homeostatic balance. Specifically, orexinergic cells of the hypothalamus constitute a feedback loop with monoaminergic and cholinergic transmitter systems of the ascending reticular activating system to facilitate wakefulness needed to carry out various adaptive and appetitive behaviours (Lopez et al., 2010; Lopez-Aumatell et al., 2011; del Cid-Pelitero, & Garzon, 2011; Yamanaka, Muraki, Tsujino, Goto, & Sakurai, 2003). Additionally, inhibitory GABAergic input from ventrolateral preoptic area (VLPO) (Martinez et al., 2002) and the basal forebrain onto orexin neurons serve to facilitate sleep, while excitatory glutamatergic input results in behavioural arousal (Henny & Jones, 2006). These findings are supported by animal research having demonstrated that hypocretin-1/orexin neurons are activated in the hypothalamus when rats are awake and active (Yoshida et al., 2001; Martinez, Smale, & Nunez, 2002; Estabrooke et al., 2001).

Orexins are also implicated in the regulation of other behaviours that serve to maintain homeostatic balance through the promotion of wakefulness (Yoshida, McCormick, Espana, Crocker, & Scammell, 2006) and motor control (Zhang et al.,

2011), including reward-seeking (reviewed in Kodadek & Cai, 2010), sexual arousal, and copulatory performance (Gulia, Mallick, & Kumar, 2003). Orexins also serve to regulate the brain-gut axis to promote arousal and digestive functions for foraging and consummatory behaviour (Kotz, 2006; Baird et al., 2009; Thorpe & Kotz, 2005; reviewed in Kirchgessner, 2002; Bulbul, Babygirija, Zheng, Ludwig, & Takahashi, 2010).

Of particular interest is evidence of the role of orexin A and the OX_2R in the modulation of anxiety-like behaviour and activation of the HPA axis (Chang et al, 2007; Li et al., 2010; Nollet et al., 2011). Administration of orexin A significantly increases anxiety-like behaviours (Suzuki at al., 2005), by inducing neuronal activation in structures of the HPA axis as evidenced by fos-like immunoreactivity in the PVN of the hypothalamus and central amygdaloid nucleus (Sakamoto, Yamada, & Ueta, 2004; Kuru et al., 2000) and increased plasma levels of stress hormones corticotrophin releasing factor (CRF), adrenocorticotropic hormone (ACTH), and corticosterone (Al-Barazanji, Wilson, Baker, Jessop, & Harbuz, 2001; Ida et al., 2000).

Research indicates that orexin neurons from the LHA innervate the PVN, a structure with widespread connections to the extended amygdala, and widely known to be involved in the regulation of anxiety-like behaviour (Li et al., 2010), as well as making extensive efferent connections within the sympathetic nervous system (Geerling, Mettenleiter, & Loewy, 2003). Orexin cells have been found to constitute fifty percent of the neurons making multisynaptic connections from the medial preoptic nucleus/lateral

hypothalamus to the medial prefrontal cortex and the heart, forming a circuit regulating negative affect and sympathetic outflow (Krout, Mettenleiter, Karpitskiy, Nguyen, & Loewy, 2005).This stimulation of the HPA axis by orexin-A is modulated by CRF and opioid systems (Ida et al., 2000; Li et al., 2010; Johnson et al., 2010) and serves to maintain vigilance and attention to potentially threatening environmental cues (Furlong, Vianna, Liu & Carrive, 2009), and to prepare the body for fight-or-flight (Zhang, Zhang, Sakurai, & Kuwaki, 2009).

Anxiety can be induced in the laboratory setting in rats by stress procedures considered 'natural' or 'unnatural', e.g., inescapable foot shock (Rod et al., 2012), chronic restraint stress (Chiba et al., 2012), social-defeat/overcrowding, chronic subordinate colony housing (Slattery et al., 2012), the odour of predators (Masini et al., 2010), vestibular stress (Markia, Kovacs, & Palkovits, 2008), early sibling deprivation (Li et al., 2008), maternal separation (Plotsky et al., 2005), anorexia (Kinzig & Hargrave, 2010), prenatal stress (Wilson, Vazdarjanova, & Terry, 2012), caloric restriction (Levay, Govic, Penman, Paolini, & Kent, 2007), and postnatal exposure to methamphetamine (Hruba, Schutova, & Slamberova, 2012) and cocaine (Kohtz, Paris, & Frye, 2010) to name a few.

While a clear threat to a rat, such as a predator or threatening conspecific, will reliably induce defensive behaviours related to fear such as defensive attack, back defence and flight, unspecific or potential danger will induce behaviours related to anxiety, such as risk assessment, vigilance, freezing behaviour and the production of

ultrasonic calls (Blanchard & Blanchard, 1989; Blanchard, Blanchard, Rodgers & Weiss, 1990). Twenty-two kilohertz ultrasonic vocalizations in particular represent a measure of negative affective state in the rat (reviewed in Brudzynski, 2001). These aversive calls are used by an animal to express the anticipation of negative contact and to alarm conspecifics. Such alarm calls are regulated by the medial cholinoceptive strip, a group of structures innervated by cholinergic fibres originating from the laterodorsal tegmental nucleus. These structures extend rostrally through areas including the anteromedial hypothalamus, the preoptic area, the bed nucleus of the stria terminalis, ventral pallidum, and lateral septum (reviewed in Brudzynski, 2001). The ascending mesolimbic cholinergic system innervates these structures, and the release of acetylcholine at the terminals reliably initiates aversive ultrasonic vocalizations. These calls can be induced to activate these neurons pharmacologically by presynaptic administration of glutamate to the laterodorsal tegmental nucleus, or postsynaptically via application of a cholinomimetic to the terminal fields located throughout the medial cholinoceptive vocalization strip (Reviewed in Brudzynski, 2001).

Administration of carbachol, an acetylcholine agonist, into the medial preoptic area of the hypothalamus has been shown to reliably induce 22 kHz aversive calling (Brudzynski, 1994). This region is densely innervated by neurons that synthesize and secrete orexin (Marcus et al., 2001), in addition to demonstrating high levels of orexin type-1 receptor expression, which bind orexin-A exclusively (Sakurai et al., 1998). Therefore, it is speculated that aversive calling in the rat may be modulated by orexingeric stimulation of the medial preoptic area.

The goal of the present study is to investigate the role of orexin-A on anxiety-like behaviour in the rat, and to determine if this neuropeptide is involved in the communication of negative affective state as expressed through the emission of 22 kHz ultrasonic vocalizations. It is hypothesized that intracerebral injection of orexin A will induce species-typical anxiety-like behaviour with concomitant emission of aversive 22 kHz alarm calls.

2. Methods

2.1 Animals

Thirty-eight adult out bred male Long Evans rats (Charles River Laboratories, Quebec) between 275-465 g of body weight were used for this study. Animals were housed in Brock University's Mammal Facility, in translucent polycarbonate cages (460 mm × 250 mm) with dust-free corn cob bedding, a dark plastic burrow for hiding, paper towel for nesting and aspen blocks for enrichment. Water and standard pellet rat chow (Harlan, WI, USA) were available ad libitum. The housing room was temperature and light controlled on a 12:12 h dark-light cycle (lights on at 08:00 h). Rats were housed in pairs before surgery and transferred to individual cages postoperatively.Surgeries were performed no sooner than four days after arrival, and all rats were handled daily to habituate them to the experimenter.

Animals were provided with veterinary care as required. All research was conducted in accordance with the *'Guide to the Care and Use of Experimental Animals'* mandated by the Canadian Council on Animal Care (Vol.1, 2nd ed; *CCAC Guide to Care and Use of Experimental Animals*, 1993)**,** and approved by the Brock University Animal Care and Use (AUPP 11-09-03).

2.2 Stereotaxic surgery and cannulation

The rats were anesthetised with Isoflurane (5% induction, 2% maintenance) and mounted on the stereotaxic frame (David Kopf Instruments, CA, USA) under aseptic conditions. As the medial preoptic area (MPOA) of the hypothalamus is innervated by

lateral hypothalamic neurons that synthesize and secrete orexin, and demonstrates high levels of orexin type-1 receptor expression (Marcus, Aschkensai, Lee, Chemelli, Saper, Yanagisawa & Elmquist, 2001; Kolaj, Coderre & Renaud, 2008), the MPOA was the target site for intracerebral administration of the drug. Sixteen millimetre long stainless steel guide cannulae (23 gauge, Becton Dickinson & Co., NJ, USA) were implanted unilaterally 1.0 mm above the medial preoptic area (7.7 mm anterior, 0.5 mm lateral, 7.2 mm ventrally from bregma) according to stereotaxic atlas (Paxinos & Watson, 1977). Guide cannulae were secured to the skull by stainless steel screws and dental acrylic (DenPlus, Longueuil, QC). A stainless steel stylet was subsequently inserted into the guide cannulae to prevent occlusion. After implantation of the guide cannulae, the incision was either closed with sterile surgical silk suture (Ethicon) or covered with dental acrylic, a novel technique endorsed by facility veterinary staff.

Animals were given one week to recover from surgery, and handled only for regular cage maintenance and postoperative care. Once rats had recovered, they were habituated to the open field apparatus and injection procedures once per day for three days before commencing the study. All animals were transferred to the acoustic recording laboratory where they were gently handled and received mock injections, and were placed in the open field testing arena for 10 minutes. Experimentation was conducted during the ‘light’ phase and only those animals deemed to be in good health were included in the experiment.

2.3 Microinjections and drugs

Pharmacological agents were dissolved in saline on the day of each injection, and administered according to a within subjects design. Drugs administered consisted of 4 µg/ 0.5 µl of orexin A (ORX) (Abcam, Cambridge, MA) a moderate dose in relation to previous research(Kuru et al., 2000; Gulia et al., 2003; Li et al., 2010), or 1 µg/ 0.5 µl of carbamylcholine chloride (carbachol, CCh) (Sigma, St. Louis, MO). Administration of carbachol served as a comparison group for 22 kHz ultrasonic calling induced by orexin, as this acetylcholine agonist reliably induces aversive calls from the medial preoptic area (Brudzynski, 1994). Rats were gently restrained during intracerebral injection to minimize distress to the animal and ensure quick and efficient injection. Stylets were removed and the injection cannulae and opening of the guide cannulae were sterilized with 70% alcohol before each injection. The drug or the vehicle (0.9% NaCl, Baxter Corp, ON, Canada) was injected (0.5 µl) through an injecting cannula (30 gauge, Small Parts Inc, Miami, FL), which protruded 1.0 mm below the guide cannula. A glass constant rate microsyringe (Hamilton Co., Reno, NV) was used to deliver the drug or vehicle at a rate of approximately 15 nl/s, with a period of 30 seconds between pre-treatment and injection. Counterbalanced double injections (saline-saline, saline-carbachol, orexin-saline, orexin-carbachol) were administered once per day with a 5 day wash out period between injections.

2.4 Ultrasonic vocalization recording

All vocalizations emitted during each 10 minute post injection testing session were recorded via a condenser microphone (model CM16/CMPA, Avisoft Bioacoustics,

Berlin, Germany) that was suspended 20 cm from the floor of the testing arena. On average, the distance between the rat and the microphone was approximately 15 cm. Vocalizations were recorded using Avisoft Bioacoustics software (Avisoft Recorder NIDAQMX) and stored on the hard drive of a PC computer (Dell Precision 390) and later saved to DVD for analysis. The acoustic files were analyzed off-line using Avisoft SAS-Lab-Pro program (with a filter of 0 - 250 kHz and FFT with resolution of 1.95 kHz).

2.5 Sonographic analysis of USVs

Twenty-two kilohertz (kHz) vocalizations typically occur in the range of 18-32 kHz, with a mean peak frequency of 22 kHz. Alarm calls of this nature are emitted in a series of calls with a narrow bandwidth (1 - 6 kHz) and a long duration (300 - 2000 ms) (Reviewed in Brudzynski, 2001).

The first and the last 20 calls recorded during the 10-minute testing session were analyzed for the following parameters: peak frequency (kHz), call duration (ms), and call bandwidth (kHz). All calls were categorized by average peak frequency as either 50-kHz calls (defined as 36 kHz or above), or as 22 kHz calls (defined as 35 kHz or below). Calls were further categorized by type as either flat or frequency modulated according to sonographic characteristics. Finally, the total number of 22 kHz ultrasonic vocalizations emitted during each testing session was counted for each rat.

2.6 Spontaneous locomotor behaviour, autonomic arousal

After receiving a microinjection of drug and/or vehicle into the MPOA, rats were placed into a square open field (43.18 cm x 43.18 cm x 20.32 cm) Digiscan Animal Activity Monitor (Columbus Instruments, OH, USA) equipped with a grid of infrared light beams (4 beams per wall) for 10 minutes. After each test, the open field was cleaned with 0.5% hydrogen peroxide (Virox Technologies, Oakville, ON). Ambulation and horizontal movements were electronically recorded as the number of infrared beam crosses. Horizontal movements included any movement recorded other than rearing. Ambulation included walking bouts, i.e., when only successive infrared beams were broken by the animal. Autonomic arousal was assessed by the number of fecal boli deposited and incidence of urination produced during each 10 minute testing session.

2.7 Histological injection localization

On the last day of behavioural testing, all animals were deeply anaesthetized with an overdose of sodium pentobarbital (120 mg/kg). Animals were then transcardially perfused with 10% formalin solution, and brains removed and fixed in formalin for at least 48 hours. The brains were blocked and serial coronal sections (60 μm) were prepared using a freezing microtome (Hacker Instruments, NJ, USA). Sections were air-dried and stained in a water solution of thionin, differentiated in three increasing concentrations of ethyl alcohol, then cleared with xylene. Stained sections were covered with Permount (Fisher Scientific, NJ, USA), cover slipped, dried and analyzed to identify injection sites according to the rat brain atlas (Paxinos & Watson, 1977).

2.8 Statistical Analysis

Statistical comparisons were performed using repeated measured analysis of variance (ANOVA), chi square test of independence, or correlation, using IBM SPSS Statistics (IBM, Armonk, New York). F tests for simple effects, Least Squares Difference, and paired t-tests were used for *post hoc* analysis where appropriate. An alpha level of $p \leq 0.05$ (two-tailed) was used to determine significance. Statistics for unequal variances and corrected degrees of freedom are reported for any analyses violating the assumption of homogeneity of variance. Data are presented as mean + SEM.

Due to the experimental limitations, the resulting low and variable sample size (n) across injection conditions did not meet the statistical assumptions (i.e., normality, homogeneity of variance, etc.) typically required of parametric statistics. As a result, all conclusions derived from the conducted parametric statistics should be interpreted with caution. In light of these stated limitations, the parametric tests utilized provide an estimation of the probability that these effects exist outside of the collected sample.

3. Results

3.1 Cannula placement

The target site for injector cannula placement was the MPOA. Injection sites were localized on the frontal stereotaxic plane as either MPOA, or anterior hypothalamic area (AHA). All other locations were grouped together and treated as a negative control (OUT). In total, cannulae placements were localized as follows: MPOA (n = 7), AHA (n = 6), OUT (n = 15), and not available N/A (n = 18), see Figure 1a & 1b. Brain slice preparations for 18 animals were damaged or compromised and cannulae placements could not be localized, therefore cannulae placements are not available.

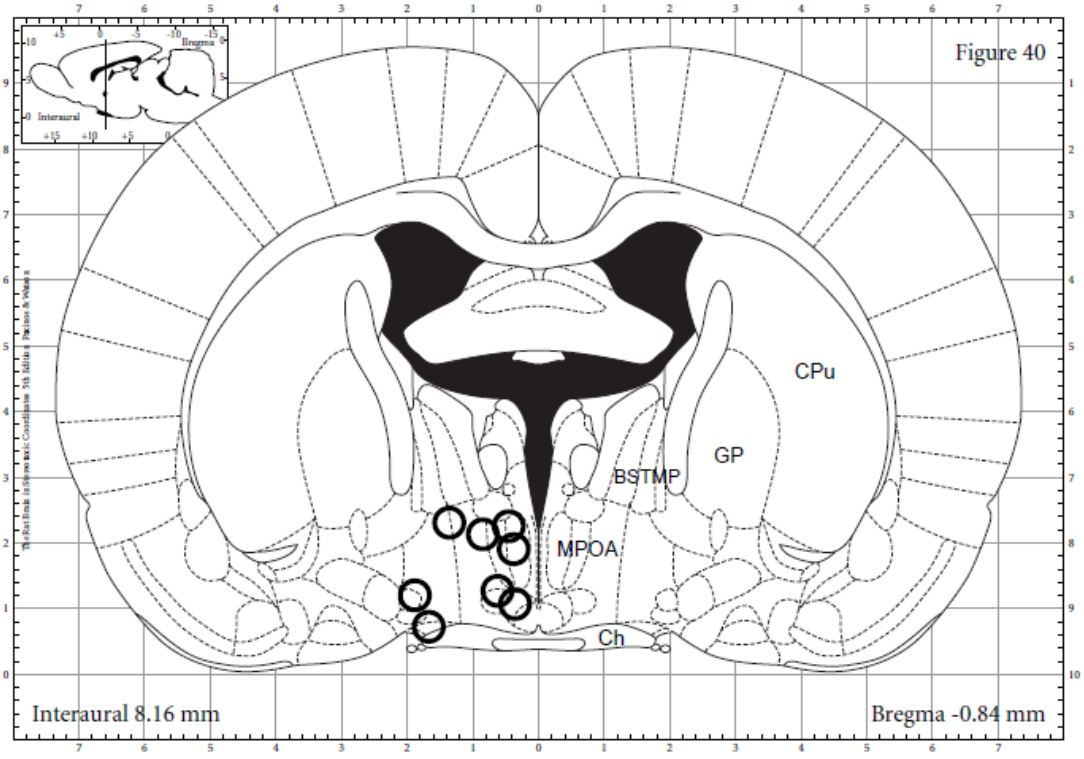


Figure 1a. Intracerebral localization of injection sites on coronal brain cross-sections (interaural 8.16 mm, Bregma -.084 mm) according to Paxinos & Watson (2005). Black circles indicate those animals that did not vocalize subsequent to intracerebral administration of carbachol. Rats with injections sites localized anterior/posterior to the hypothalamic region are not shown. Abbreviations: BSTMP: Bed Nucleus of the

Stria Terminalis, Ch: Optic Chiasm, CPu: Caudate/Putamen, GP: Globus Pallidus, MPOA: Medial Preoptic Area.

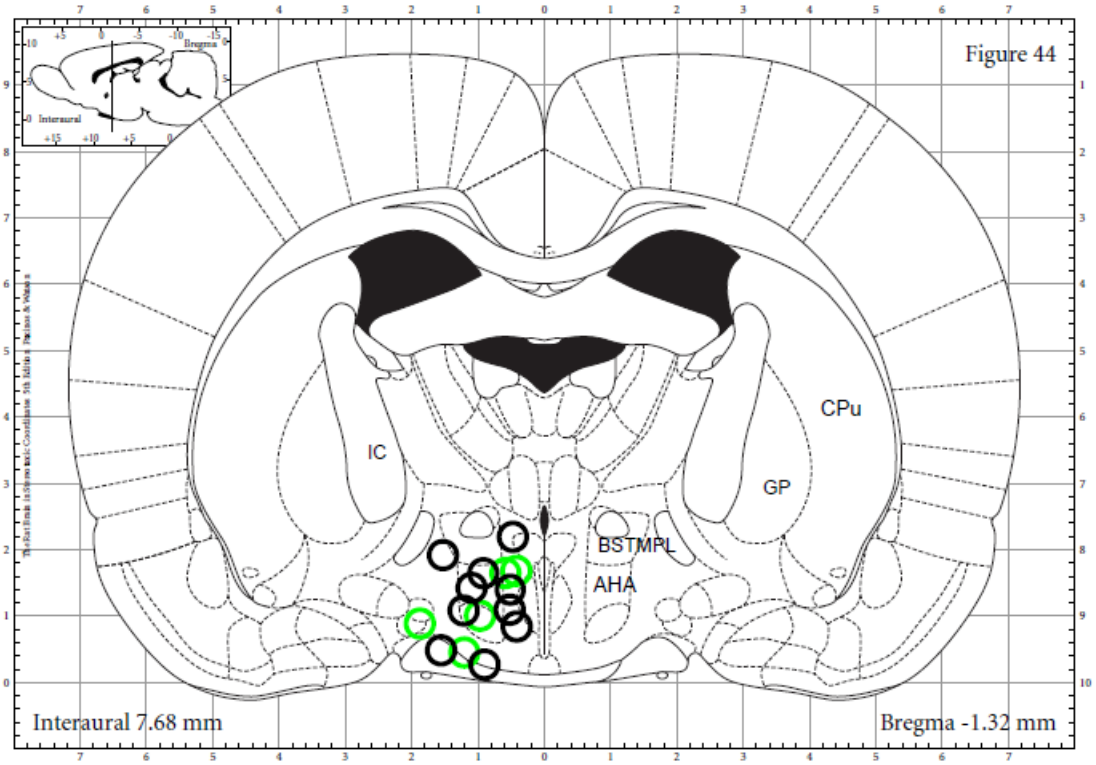


Figure 1b. Intracerebral localization of injection sites on a coronal brain cross section (interaural 7.68 mm, Bregma -1.32 mm) according to Paxinos & Watson (2005). Green circles indicate those animals that vocalized subsequent to intracerebral administration of carbachol. Rats with injections sites localized anterior/posterior to the hypothalamic region are not shown. Abbreviations: AHA: Anterior Hypothalamic Area, BSTMPL: Bed Nucleus of the Stria Terminalis Posterolateral, CPu: Caudate/Putamen, GP: Globus Pallidus, IC: Internal Capsule.

3.2 Effects of microinjections of orexin-A and carbachol on the number of 22 kHz aversive calls.

Descriptive statistics for average number of 22 kHz ultrasonic vocalizations induced by intracerebral injections indicate that intracerebral administration of saline and orexin did not induce 22 kHz aversive ultrasonic calls at the studied dose, while carbachol did induce such vocalizations.

Table #1 shows means and standard deviations of the average number of 22 kHz ultrasonic vocalizations as a function of injection conditions, and Figure 2 depicts these data in graphical form.

Table 1.
Descriptive statistics of the average number of 22 kHz ultrasonic vocalizations induced as a function of drug injection condition.

Injection Condition	Mean Number of Calls (SE)	*N*
Sal-Sal	0 (0)	25
Sal-Orx	0 (0)	26
Sal-CCh	4.79 (2.77)	25
Orx-CCh	0.08 (0.08)	10

Note: Sal: saline; Orx: orexin-A; CCh: carbachol

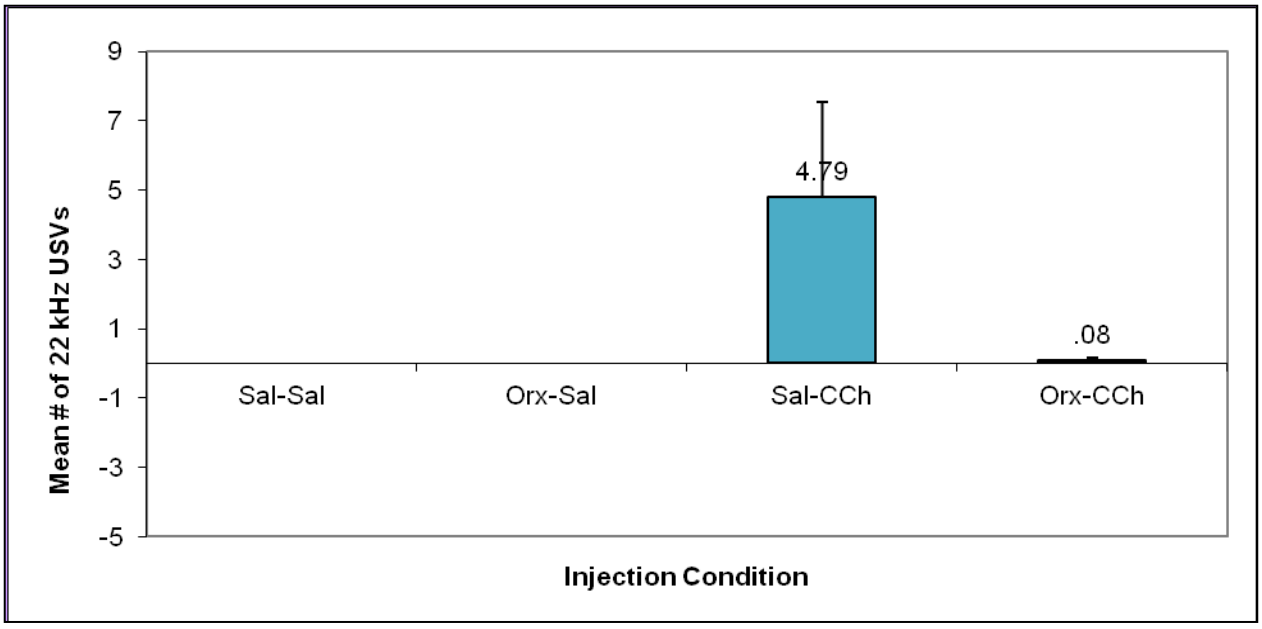


Figure 2. Number of 22 kHz ultrasonic vocalizations induced by intracerebral injection (saline-saline, orexin-saline, saline-carbachol, and orexin-carbachol). The data show that

orexin does not appear to induce 22 kHz ultrasonic vocalizations; carbachol-induced vocalizations and response may be inhibited by pre-treatment of orexin.

The acoustic parameters of 22 kHz ultrasonic calls, or alarm calls, that were induced by intracerebral administration of saline-carbachol were found to be species typical. Table 2 provides the descriptive statistics of average call parameters of the 22 kHz ultrasonic vocalization induced by intracerebral administration of saline-carbachol. Figure 3 provides sample sonograms of 22 kHz ultrasonic vocalizations induced by injection of saline-carbachol. The 22 kHz ultrasounds had a stable sound frequency lasting approximately 1000 ms, with a very narrow bandwidth.

Table 2.
Descriptive statistics of call parameters of the 22 kHz ultrasonic vocalization induced by intracerebral administration of saline-carbachol.

	Average Number of Calls Emitted (*n*)	Duration (Seconds)	Bandwidth (kHz)	Peak Frequency (kHz)
Mean	31	1.74	4.6	22.13

Note: Five animals out of 34 emitted 22 kHz ultrasonic vocalizations subsequent to administration of the saline-carbachol, while the remaining 29 animals did not vocalize after such administration.

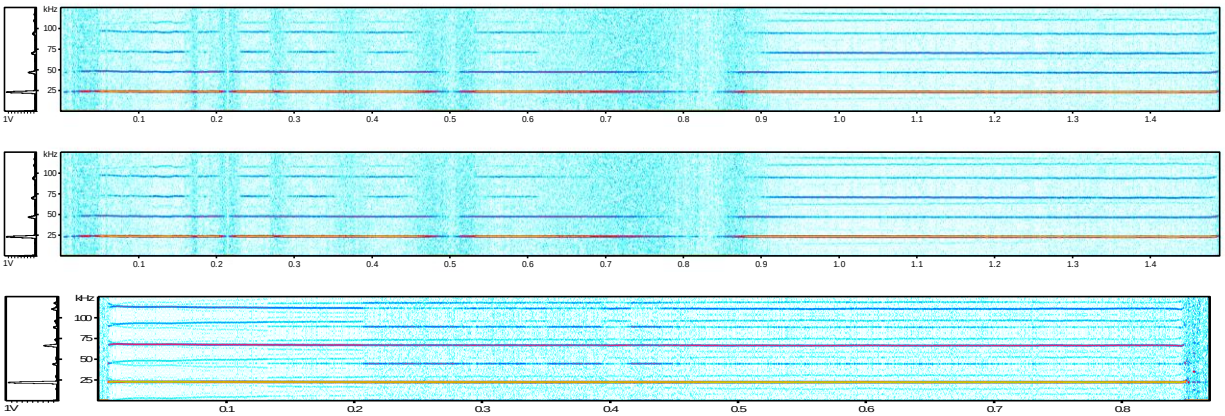


Figure 3. Examplary sonograms of 22 kHz ultrasonic vocalizations induced by intracerebal injection of saline-carbachol. Time scale on the x-axis is in seconds, and the y-axis in kilohertz (0 - 125 kHz). The peaks in the rectangles on the left hand side

indicate peak frequencies. The duration of these vocalizations is from 0.8 to 1.5 seconds, with the fundamental frequency 24 - 25 kHz.

3.3 Effects of microinjection of orexin-A and carbachol into the MPOA and AHA on the number of 22 kHz aversive vocalizations.

The average number of 22 kHz ultrasonic vocalizations induced by intracerebral injection was further analyzed as a function of injection localization. Table 3 provides the means and standard errors of the number of 22 kHz ultrasonic vocalizations induced by intracerebral injections; Figure 4 shows these data in graphical form. These data indicate that intracerebral administration of orexin-A and saline did not induce aversive calls. Carbachol, on the other hand, did induce 22 kHz calls, but only when administered to the medial preoptic area or anterior hypothalamic area.

Table 3.
Descriptive statistics of mean of number of 22 kHz ultrasonic vocalizations induced by orexin, carbachol and saline control as a function of localization.

	MPOA		AHA		OUT	
Injection Condition	Mean # of Calls (SE)	*n*	Mean # of Calls (SE)	*n*	Mean # of Calls (SE)	*n*
Sal-Sal	0 (0)	6	0 (0)	6	0 (0)	10
Orx-Sal	0 (0)	8	0 (0)	5	0 (0)	11
Sal-CCh	4.0 (4.0)	8	20.2 (16.08)	5	0 (0)	12
Orx-CCh	0 (0)	3	0 (0)	3	0 (0)	4

Note: MPOA: medial preoptic area; AHA: anterior hypothalamic area; OUT: any other location outside of the MPOA or AHA.

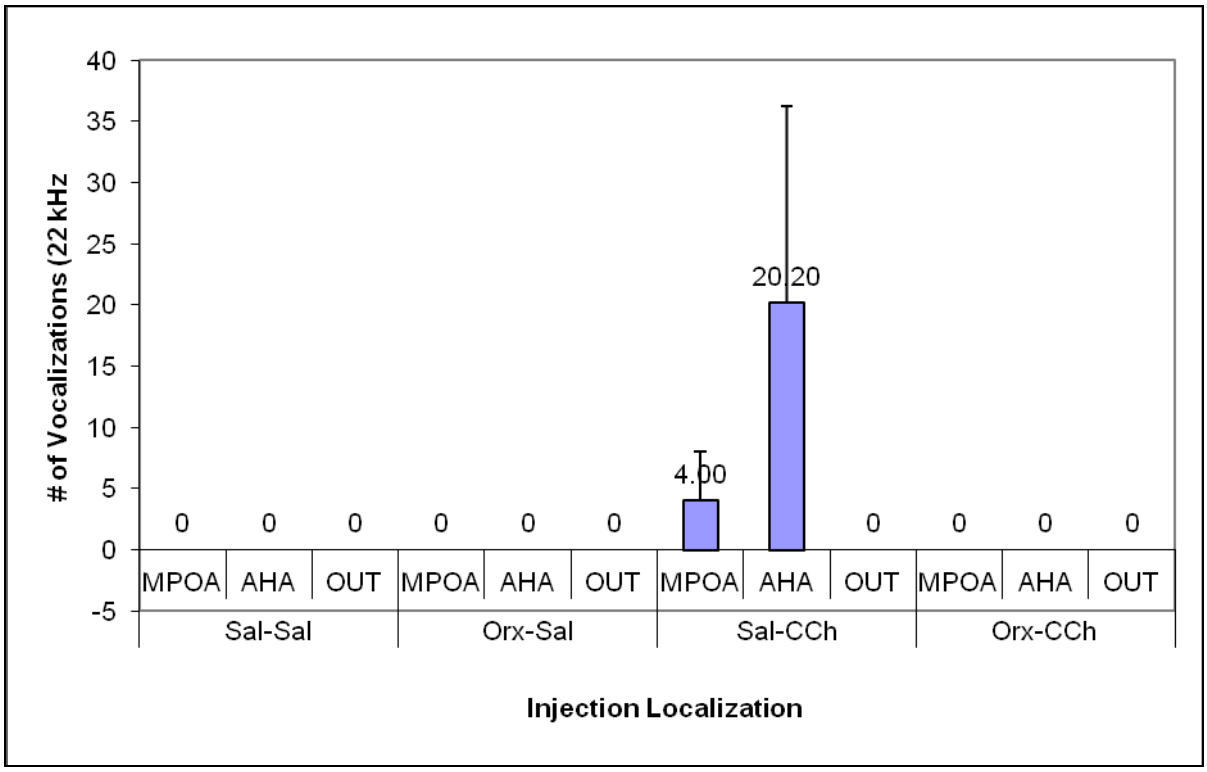


Figure 4. Number of ultrasonic vocalizations induced by intracerebral injection as a function of injection localization. These data demonstrate that saline and orexin did not induce 22 kHz ultrasonic calls. Carbachol does induce aversive calls, but only when administered to MPOA and AHA, although this difference was not found to be significant ($p > .05$).

An independent measures t-test was conducted to determine the effect of localization on carbachol induced 22 kHz vocalizations. It was found that there was not a significant effect of localization on the number of calls induced by carbachol, $t\ (11) = -1.21$, $p = .25$. Therefore, saline-carbachol does appear to induce more 22 kHz ultrasonic vocalizations above other treatment groups, although in this case the effects of this drug on 22 kHz ultrasonic calling rate are not significantly different when administered to the MPOA or AHA.

3.4 Effects of microinjection of orexin-A and carbachol on autonomic arousal

Urination

A chi-square test of independence was conducted to determine the effect of intracerebral injection on urination (see Table 4 for descriptive statistics). It was found that urination and injection condition are independent of one another, χ^2 (3) = 4.21, p = .24. Therefore, the incidence of urination does not depend on type of agent administered to the brain (orexin-A, carbachol, or saline - see Figures 5a and 5b). Although injection condition is independent of urination, examination of the standardized residuals indicates a higher incidence of urinating animals injected with orexin-saline than the others, whereby the observed occurrence of urination is twice that of the expected rate.

Table 4.
Percentage of incidence of urination in rats as a function of intracerebral injection condition.

Urination	Sal-Sal	Orx-Sal	Sal-CCh	Orx-CCh	All Injection Conditions
NO	94.6%	83.8%	94.3%	94.6%	91.8%
YES	5.4%	16.2%	5.7%	5.4%	8.2%

Table 5.

Results table for Chi-Squared test of independence of urination as a function of injection condition

		Sal-Sal	Orx-Sal	Sal-CCh	Orx-CCh
No	Count	35	31	33	35
	Expected Count	34.0	34.0	32.1	34.0
	Residual	1.0	-3.0	0.9	1.0
	Std. Residual	0.2	-0.5	0.2	0.2
Yes	Count	2	6	2	2
	Expected Count	3.0	3.0	2.9	3.0
	Residual	-1.0	3.0	-0.9	-1.0
	Std. Residual	-0.6	1.7	-0.5	-0.6

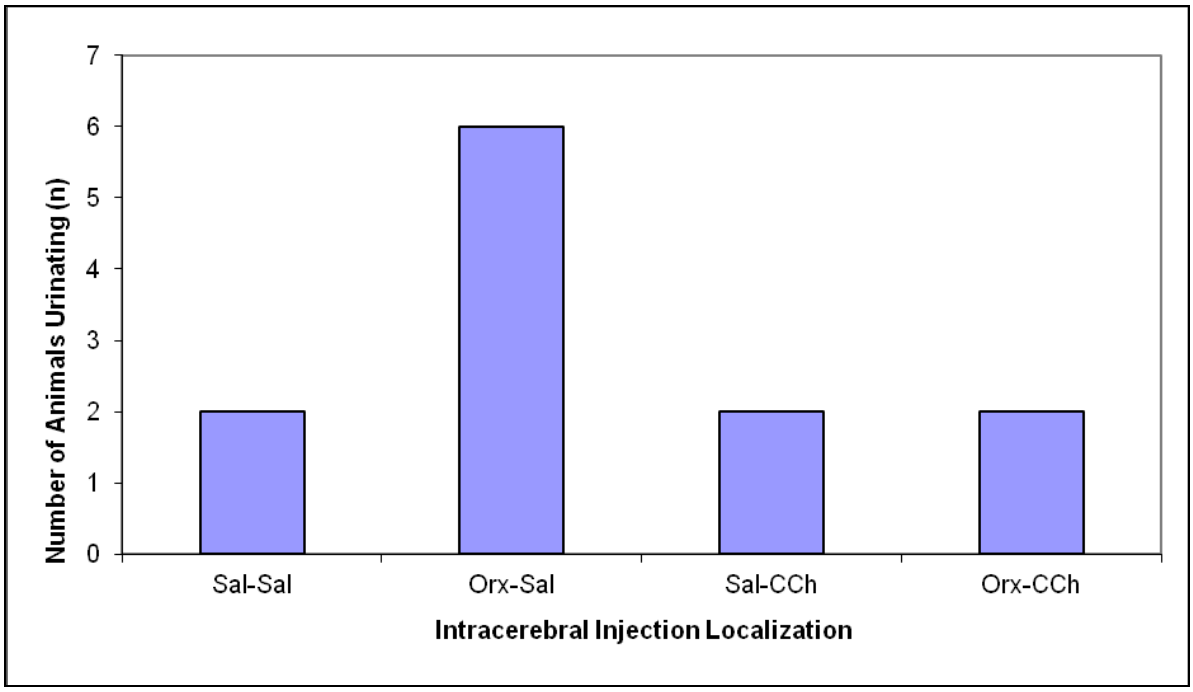


Figure 5a. Number of animals urinating as a function of intracerebral injection condition.

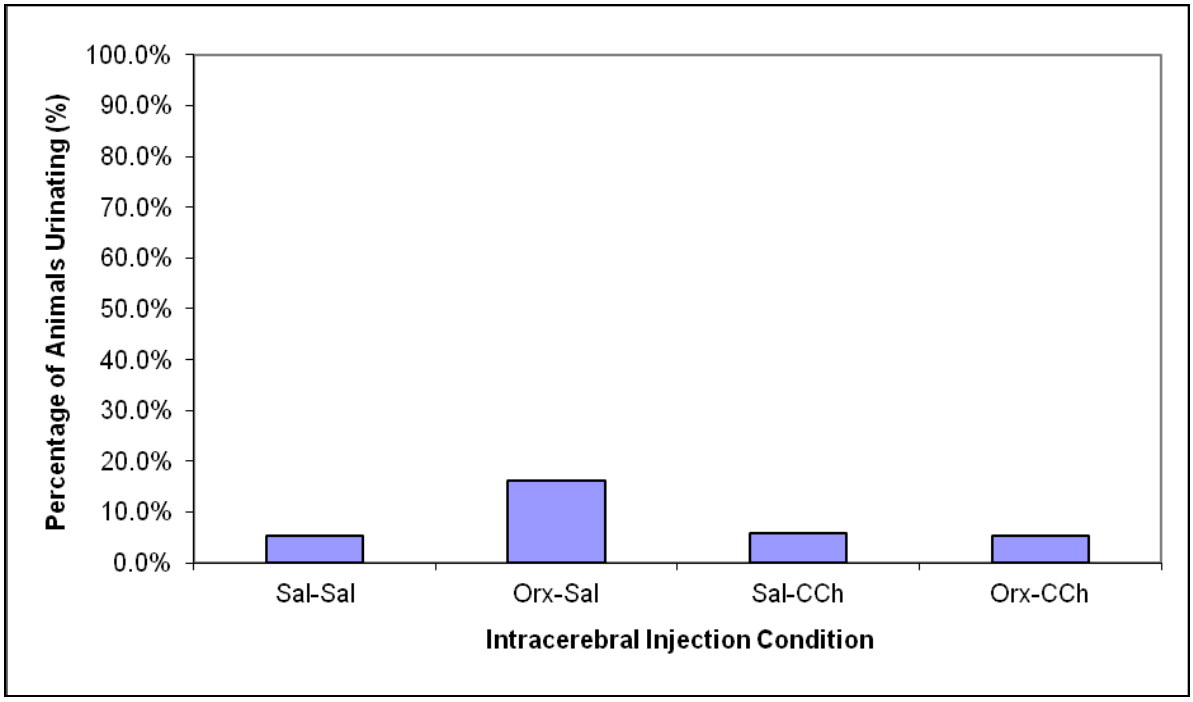


Figure 5b. Percent of animals urinating after intracerebral injection of drug.

A chi-square test of independence was conducted to determine the effect of injection localization on urination. It was found that urination and localization are independent of

one another, χ^2 (2) = .58, p = .75. Therefore, the incidence of animal urination did not depend on where the orexin, carbachol, or saline control was administered in the brain.

Defecation

A mixed model 3 (localization [MPOA, AHA, OUT]) x 3 (injection condition [saline-saline, orexin-saline, saline-carbachol) ANOVA was conducted to determine the effect of intracerebral injection condition and injection localization on the number of fecal boli produced in the open field (see Table 6 for descriptive statistics). Results indicate a significant main effect of condition, F (2, 44) = 3. 47, p = .04, but no main effect of localization, or interaction between localization and injection condition. Table 7 presents the ANOVA results and Figure 6 provides a graphical depiction of the data.

Three repeated measures *t*-tests were conducted to examine the main effect of injection condition on defecation. Results indicate that the number of fecal boli produced by intracerebral administration of orexin-saline and saline-carbachol are significantly different from that of saline-saline (t (36) = -2.16, p = .04, t (34) = 3.67, p = .001 respectively). Intracerebral administration of orexin-saline and saline-carbachol induced a higher rate of defection than the saline control. However, there was no significant difference between the number of fecal boli produced by injection of orexin or carbachol, t (34) = - 1.21, p = 0.24.

Overall, it can be seen that orexin and carbachol administered intracerebrally significantly increased the number of fecal boli deposited above saline.

Table 6.

Descriptive statistics of the average number of fecal boli produced in the open field after administration of orexin, carbachol and saline control as a function of localization

Injection Condition	Localization	Mean	SE	*N*
Sal-Sal	MPOA	0.00	0.00	8
	AHA	0.00	0.00	6
	OUT	0.00	0.00	11
Orx-Sal	MPOA	0.00	0.00	8
	AHA	1.83	3.13	6
	OUT	1.27	2.87	11
Sal-CCh	MPOA	.63	1.41	8
	AHA	1.00	2.00	6
	OUT	1.27	1.74	11

Table 7. *Results of mixed model 3 (localization) x 3 (injection condition) ANOVA conducted to determine the effect of intracerebral injection and injection localization on the number of fecal boli produced in the open field*

	df	*F*	*η*	*P*
Within Effects				
Injection Condition	2	3.47	.14	.04
Injection Condition X Localization	4	0.82	0.07	0.52
Error	44			
Between Effects				
Localization	2	0.87	0.07	0.43
Error	22			

Note: * Indicates significant difference from saline control, $p < 0.05$.

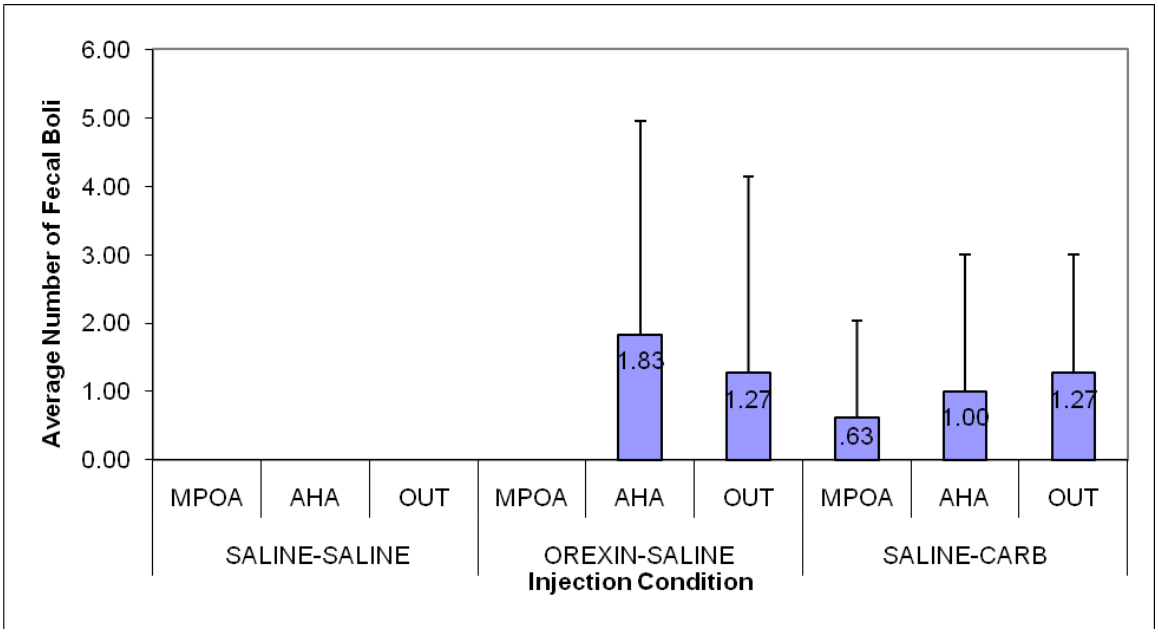


Figure 6. Average number of fecal boli produced in the open field after intracerebral injection of orexin, carbachol and saline control as a function of localization.

Overall Arousal

A chi-square test of independence was conducted to determine the effect of injection condition on overall autonomic arousal (urination: yes/no + defecation: yes/no). Results indicate that overall arousal is dependent on injection condition, χ^2 (6) = 18.22, p = .006. Therefore, overall autonomic arousal in the rats as measured by incidence of urination and/or defecation depends on the drug administered intracerebrally to the brain. Analysis of the standardized residuals indicates that a greater number of animals intracerebrally injected with saline-carbachol were found to urinate or defecate than would be expected (std. residual = 2.7), as did the administration of orexin-saline injected animals (std. residual = 1.2 - See Table 8).

Table 8.
Results of chi-square test of independence of overall arousal as a function of injection condition.

		Sal-Sal	Orx-Sal	Sal-CCh
No Urination or Defecation	Count	34	27	20
	Expected Count	28.9	28.9	27.3
	Std. Residual	1.0	-.4	-1.4
Urination or Defecation	Count	3	7	13
	Expected Count	6.6	6.6	6.2
	Std. Residual	-1.4	.2	2.7
Both Urination and Defecation	Count	0	3	2
	Expected Count	1.5	1.5	1.4
	Std. Residual	-1.2	1.2	.5

Note: No urination or defecation: rat did not urinate or defecate in open field subsequent to intracerebral administration of drug.

3.5 Locomotor behaviour

Horizontal Movement

A mixed model 3 (localization [MPOA, AHA, OUT]) x 3 (injection condition [saline-saline, orexin-saline, saline-carbachol) ANOVA was conducted to determine the effect of injection condition and localization on horizontal movement (# of infrared beam crosses - see Table 9 for descriptive statistics). Sphericity could not be assumed for condition (Mauchly's W (2) = .32, p = .06), therefore the Greenhouse-Geisser correction

to degrees of freedom was applied. Results of the ANOVA (see Table 10) indicate that there is not a significant main effect of injection condition, $F^{G\text{-}G}$ (1.19, 7.15) = .05, p = .86. Equal variances are assumed for localization (Levene's test p values > .05). Results indicate that there was not a main effect of localization, F (2, 6) 1.52, p = .29. There was no significant interaction between injection condition and localization, $F^{G\text{-}G}$ (2.38, 7.15) = .23, p = .83. Although the effect of injection condition on horizontal movement was not significant, it was observed that rats administered saline-orexin to the AHA produced the lowest rate of horizontal movement. Figure 8 depicts these results below.

Table 9.
Descriptive statistics of horizontal movement following injection of drug as a function of localization.

Injection Condition	Localization	Mean	SE	*n*
Sal-Sal	MPOA	2168.00	624.982	4
	AHA	1610.00		1
	OUT	1888.00	328.894	4
Orx-Sal	MPOA	2093.25	702.169	4
	AHA	1487.00		1
	OUT	2013.25	529.290	4
Sal-CCh	MPOA	2284.50	173.667	4
	AHA	1312.00		1
	OUT	1816.25	654.688	4

Note: Mean represents that average number of infra-red light beam crosses

Table 10.

Results of mixed model 3 (localization) x 3 (injection condition) ANOVA to determine the effect of injection condition and localization on horizontal movement

	df	*F*	*η*	*P*
Within Effects				
Injection Condition	1.44	0.42	0.05	0.60
Injection Condition X Localization	2.88	0.72	0.14	0.55
Error	12.97			
Between Effects				
Localization	2	9.31	0.67	.01
Error	9			

Note: * Indicates significant difference from saline control, $p < 0.05$.

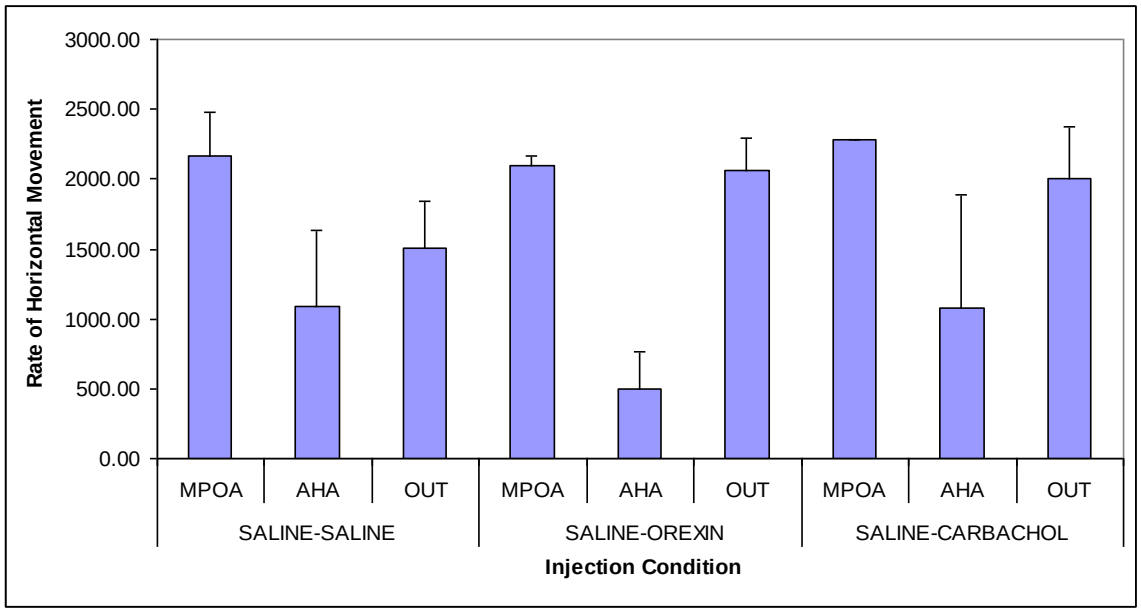


Figure 7. Rate of horizontal activity for each injection condition as a function of injection localization. The rate of horizontal activity is expressed in number of broken light beams.

Ambulation

A mixed model 3 (localization [MPOA, AHA, OUT]) x 3 (injection condition [saline-saline, orexin-saline, saline-carbachol) ANOVA was conducted to determine the effect of injection condition and injection localization on ambulation (see Table 11 for descriptive statistics). Results of the ANOVA indicate that there is a main effect of localization, F (2, 9) = 8.47, p = .01 (see Table 12). A LSD post-hoc analysis was conducted to follow up this effect and produced significant differences between AHA and MPOA, and AHA and OUT ($p < .05$). Rates of ambulation were lower in those rats intracerebrally injected into the AHA compared to the MPOA and OUT, as seen in Figure 8.

Although there was no significant main effect of injection condition, nor an interaction, it was observed that, similar to the horizontal movement results reported above, rats injected with saline-orexin had the lowest rate of ambulation compared to saline-saline and saline-carbachol when they were administered into the AHA. This observation may be important since ambulation represents better locomotor progression (walking) as compared to general activity of the organism (e.g., licking and grooming), and may, therefore, be a more meaningful indicator of behavioural inhibition and decreased locomotor behaviour.

Table 11.

Descriptive statistics of rate of ambulation following administration of orexin, carbachol, and saline control as a function of localization.

Injection Condition	Localization	Mean	SE	*n*
Sal-Sal	MPOA	1747.00	265.120	4
	AHA	854.00	427.264	3
	OUT	1199.00	269.985	5
Orx-Sal	MPOA	1644.50	248.517	4
	AHA	389.00	389.000	3
	OUT	1604.00	161.313	5
Sal-CCh	MPOA	1855.50	63.212	4
	AHA	854.00	419.508	3
	OUT	1589.00	617.499	5

Table 12.
Results of mixed model 3 (localization) x 3 (injection condition) ANOVA was conducted to determine the effect of injection condition and cannula localization on ambulation

	df	*F*	*η*	*P*
Within Effects				
Injection Condition	2	0.49	0.05	0.62
Injection Condition X Localization	4	0.62	0.12	0.66
Error	18			
Between Effects				
Localization	2	8.47	0.65	.01*
Error	9			

Note: * Indicates significant difference as compared to the saline control, $p < 0.05$.

Four one-way ANOVAs were conducted to further examine the effect of injection localization on ambulation for each injection condition. Results of the ANOVA indicate a significant effect of localization for saline-carbachol and orexin-saline, F (2, 13) = 4.64, p = .03, and F (2, 11) = 6.08, p = .02 respectively. A LSD post-hoc analysis was conducted to follow up the significant effect of saline-carbachol and orexin-saline on ambulation, and indicated a significant difference between AHA and MPOA and AHA and OUT ($p <$ 0.5). Therefore, when rats were injected with saline-carbachol and saline-orexin into the AHA, there is a significant decrease in the rate of ambulation compared to those injected into the MPOA and OUT.

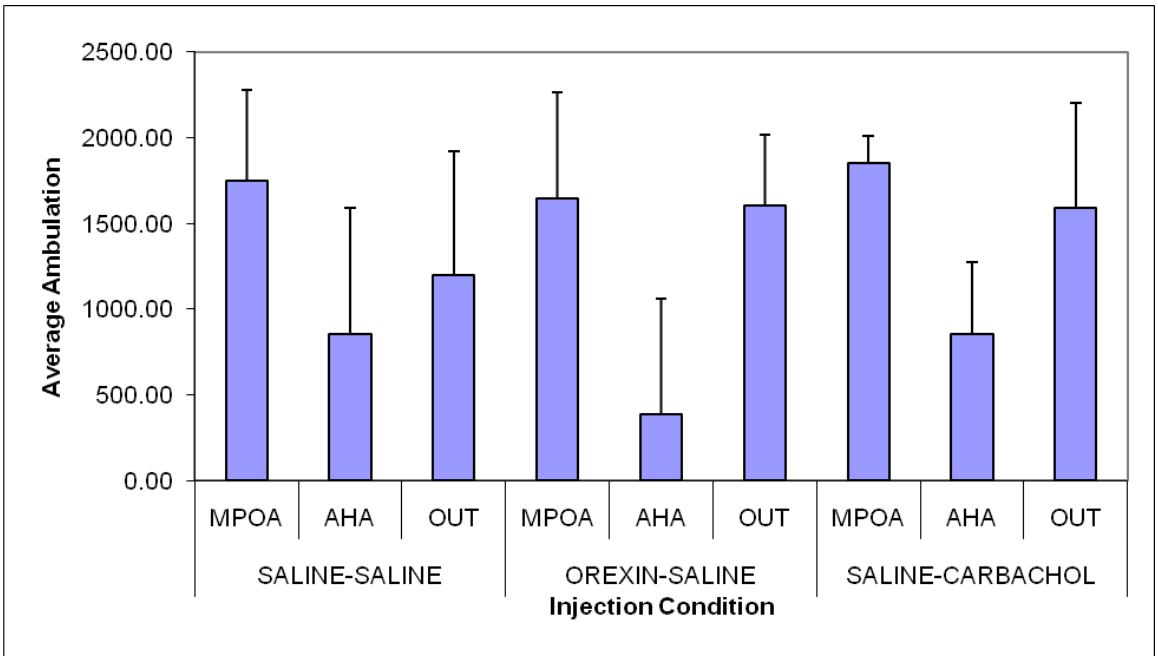


Figure 8. Rate of ambulation for each injection condition as a function of cannula location. Ambulation is expressed in the number of broken light beams.

3.6 Correlation between anxiety-like behaviours expressed by the number of fecal boli produced.

As an increased number of fecal boli deposited concomitant with reduced locomotor behaviour may be indicative of anxiety-like behaviour, correlational analyses were conducted. It was found that there was no significant correlation between the number of fecal boli produced and the rate of ambulation for the saline control, $r^2 = 0.54$ $p > .05$, orexin-saline, $r^2 = -0.16$ $p > .05$, or saline-carbachol, $r^2 = 0.36$ $p > .05$. Nor were there found to be significant correlations between the number of fecal boli produced and horizontal activity for the saline control, $r^2 = 0.57$ $p > .05$, orexin-saline, $r^2 = -0.15$ $p > .05$, or orexin-carbachol, $r^2 = 0.36$ $p > .05$.

4. Discussion

This study shows that intracerebral administration of orexin-A significantly increases autonomic arousal in the rat, as measured by increased fecal boli output. The current results are congruent with previous work demonstrating that administration of orexin-A stimulates colonic motility in the rat in a dose dependent manner (Nozu et al., 2011). The number of fecal boli deposited is used as an effective measure of emotionality, particularly autonomic arousal and anxiety in rats (Broadhurst, 1957). Stress has been shown to dysregulate the brain-gut axis and stimulate contraction of the colon and increase fecal output (Liang, Luo, Liu, Cao, & Xia, 2012; Monnikes, Schmidt, Tebbe, Bauer, & Tache, 1994). Consistent with these reactions, our results indicate that central administrations of orexin-A did activate sympathetic activity indicative of anxiety.

Despite this increase in defecation in response to administration of orexin-A, there was no negative, and expected, correlation between orexin-A administration and overall autonomic arousal or locomotor behaviour, typical of anxiety-like defensive behaviour (Williams & Russell, 1972). Although orexin-A injected animals showed a tendency to decrease locomotor behaviour, this effect did not reach significance. Taken with the increases in autonomic arousal, these data suggest that an aversive state and defensive behaviour were induced. As it has been found that defecation in the open field correlates with levels of corticosterone, but not consistently with overall locomotor behaviour (Gentsch, Lichtsteiner, & Feer, 1981; Pradhan & Arunasmitha, 1991), or ambulation (Pare, 1964; Russell, 1973), this study will rely on the significant increase in fecal boli output as a reliable marker of anxiety and recruitment of the HPA axis.

Alternatively, it may be speculated that animals remained active after the administration of orexin-A and that this behaviour reflected orexinergic promotion of wakefulness (Lopez et al., 2010; Lopez-Aumatell et al., 2011; del Cid-Pelitero & Garzon, 2011; Yamanaka et al., 2003), activation the HPA axis (Li, et al., 2010), increased postural muscle tone (Takakusaki et al., 2005), and sympathetic outflow (Geerling, Mettenleiter, & Loewy, 2003; Krou et al., 2005) relevant for preparation for fight-or-flight behaviours. Research with nocturnal and diurnal animals support such a link between orexin-A and increased locomotor behaviour, as it has been found that this neuropeptide stimulates midbrain motor areas to sustain muscle tone and promote high levels of locomotor behaviour during wakefulness (Takakusaki et al., 2005; Mileykovskiy, Kiyashchenko, & Siegel, 2002). However, the observation of the tendency for decreased locomotor activity in our study would imply that intracerebral administration of orexin-A produced localized activation of the medial preoptic/anterior hypothalamic areas.

Measurement of stress hormone corticosterone (Lim, et al., 2011) and more sophisticated measures of anxiety, like the elevated plus maze (Pawlak, Karrenbauer, Schneider, & Ho, 2012), light/dark box (Ramos, Pereira, Martins, Wehrmeister, & Izídio, 2008), open space (Ennaceur, Michalikova, & Chazot, 2006), and locomotor behaviour (center field versus thigmotaxis) (Fernandes et al., 1999; Mallo et al., 2007) could be used in the future to correlate rates of defecation with anxiety-like behaviour induced by orexin-A to better understand behaviour observed after such administration.

Although orexin-A did have an effect on autonomic measures of anxiety, it did not induce 22 kHz ultrasonic vocalizations. These ultrasounds are an antipredator adaptation, functioning as an alarm signal directed to the members of the social group about an approaching predator or potential threat (Brudzynski, 2001; Blanchard & Blanchard, 1989). Such high-pitched calls are produced when air is forced through the constricted vocal folds creating a whistle-like effect. This higher order defensive behaviour allows rats to communicate in the ultrasonic range, largely undetectable by predatory animals above the burrow system (Brudzynski, 2001; Litvin, Blanchard & Blanchard, 2007).

Twenty-two kHz ultrasonic vocalizations represent a reliable measure of negative affect in the rat, reflecting states of anxiety in anticipation of possible threats to the individual or the greater colony (Litvin et at., 2007; reviewed in, Brudzynski, 2013). Alarm calls are unambiguously interpreted by all rats as a sign of warning, and induce behavioural inhibition and freezing in the receiving animals (Brudzynski, 2013). These calls serve as an ethological transmitter or ethotransmitter which represents a species-specific signal of negative affect and anxiety.

This study used an injection dosage of 0.5 µg of orexin-A which is a relatively moderate dose as compared to other research studies, having administered between 0.3 µg to 30 µg of the drug (Kuru et al., 2000; Gulia et al., 2003; Li et al., 2010). Since orexinergic neurons increase excitability by depolarizing postsynaptic receptors, as well as exciting glutamatergic input into the lateral hypothalamus (Kolaj et al., 2008), it could

be speculated that orexin-A is not involved in the direct initiation of ultrasonic signalling. For example, unpublished data from our lab show that administration of carbachol pre-treated with a dose of orexin less than 0.05 µg resulted in unchanged 22 kHz ultrasonic calling, and indicates that the dose of orexin-A applied in the current study being 10 times higher could have inhibited the aversive calling. Administration of orexin-A in a dose dependent manner could further clarify the relationship between orexin-A and the communication of negative affect.

The present data may be indicative of the influence orexin-A may have on the hypothalamic/preoptic area and its ability to regulate internal homeostatis, sympathetic control, and wake-promoting physiological functions necessary to carry out defensive behaviour, while not stimulating the medial cholinoceptive vocalization strip. This collective group of structures constitutes of all of the areas of the midbrain and forebrain that are cholinergically innervated by the ascending cholinergic system originating from the laterodorsal tegmental nucleus (Brudzynski, 2001; 2013). As animals did not vocalize after administration of orexin-A, our data indicate that postsynaptic application of orexin-A to the terminal fields of the medial cholinoceptive vocalization strip does not activate cholinergic neurons involved in the communication of negative affect.

Future directions for research will include further investigation into a potential role of orexin-A in the control of ultrasonic vocalizations. More refined measures of autonomic arousal indicative of anxiety, including cardiorespiratory response (Zhang et al., 2009), and stress hormones (Viau, Sharma, & Meaney, 1996), could be used to

correlate observed increases in fecal boli output and urination with decreases in ambulation after administration of orexin-A. These measures could also be used to examine correlations with ultrasonic calling rate as a means to study the role of orexin-A in the communication of negative affective state.

5. Conclusions

As hypothesized, this study demonstrates that central administration of orexin-A may activate anxiety-like behaviours and autonomic arousal in the rat indicative of negative affect and a defensive state. However, under the present conditions, orexin-A does not appear to be involved in the activation of the medial cholinoceptive vocalization strip for the communication of such negative affective state via the emission of 22 kHz ultrasonic vocalizations.

References

Actnity, L., Observed, D., Pairs, I., & Sessions, R. (1998). Locomotor activity and defecation of rats observed. *Comparative and General Pharmacology*, 1179-1184.

Al-Barazanji, A., Baker, W., Jessop, D., & Harbuz, M. (2001). Central orexin-A activates hypothalamic-pituitary-adrenal axis and stimulates hypothalamic corticotrophin releasing factor and arginine vasopressin neurons in conscious rats. *Journal of Neuroendocrinology, 13,* 421-424.

American Psychiatric Association (2013). *Diagnostic and Statistical Manual of Mental Disorders* (5th ed.). Arlington, VA: American Psychiatric Publishing.

Baird, J., Choe, A., Loveland, J., Beck, J., Mahoney, C., Lord, J., & Grigg, L. (2009). Orexin-A hyperphagia: Hindbrain participation in consummatory feeding responses. *Endocrinology, 150*(3), 1202-1216. doi: 10.1210/en.2008-0293

Barlow, D. (2002). Anxiety and its disorders: The nature and treatment of anxiety and panic. United States of America: The Guildford Press.

Broadhurst, P. (1957). Determinants of emotionality in the rat. I. Situational factors. *British Journal of Psychology, 48,* 1-12.

Blanchard, R., & Blanchard, D. (1989). Antipredator defensive behaviours in a visible burrow system. *Journal of Comparative Psychology, 103*(1), 70-82.

Blanchard, D, Blanchard, R., & Rodgers, R. (1993). Pharmacological and neural control of anti-predator defense in the rat. *Aggressive Behavior, 16,* 165-175.

Blanchard, R., Blanchard, D., Rodgers, J., & Weiss, S. (1990). The characterization and modelling of antipredator defensive behaviour. *Neuroscience and Biobehavioral Reviews, 14*(4), 463-72.

Bouwknecht, J., Spiga, F., Staub, D., Hale, M., Shekhar, A., & Lowry, C. (2007). Differential effects of exposure to low-light or high-light open-field on anxiety-related behaviors: Relationship to c-Fos expression in serotonergic and non-serotonergic neurons in the dorsal raphe nucleus. *Brain Research Bulletin, 72*(1), 32-43. doi:10.1016/j.brainresbull.2006.12.009

Broadhurst, P. (1957). Determinants of emotionality in the rat. I. Situational factors. *British Journal of Psychology, 48*(1). 1- 12.

Brudzynski, S. (2013). Ethotransmission: Communication of emotional states through ultrasonic vocalization in rats. *Current Opinion in Neurobiology, 23,* 310-317.

Brudzynski, S. (2001). Pharmacological and behavioral characteristics of 22 kHz alarm calls in rats. *Neuroscience and Biobehavioural Reviews, 25*, 611-617.

Brudzynski, S., McLachlan, R., Bihari, F. & Girvin, J. (1991). Response of neurons of the rat anterior hypothalamic-preoptic area to carbachol. *Brain Research Bulletin, 26*, 929-934.

Brundin, L., Bjorkqvist, M., Petersen, A. & Traskman-Bendz, L. (2007). Reduced orexin levels in the cerebrospinal fluid of suicidal patients with major depressive disorder. *European Neuropsychopharmacology, 17,* 573-579. doi: 10.1016/j.euroneuro.2007.01.005

Bulbul, M., Babygiriya, R., Zheng, J., Ludwig, K. & Takahashi, T. (2010). Central orexin-A changes the gastrointestinal motor pattern from interdigestive to postprandial in rats. *Autonomic Neuroscience: Basic and Clinical, 158,* 24-30. doi: 10.1016/j.autneu.2010.05.009

Burt, J., Alberto, C, Parsons, P. & Hirasawa, M. (2011). Local network regulation of orexin neurons in the lateral hypothalamus. *American Journal of Physiology-Regulatory, Integrative and Comparative physiology, 30,* 572-580. doi: 10.1152/ajpregu.00674.2010

Canadian Council on Animal Care. (1993). *CCAC Guide to Care and Use of Experimental Animals*, vol.1, 2nd ed. Retrieved August 1st, 2013 from http://www.ccac.ca/Documents/Standards/Guidelines/Experimental_Animals_Vol1.pdf.

Chang, H., Saito, T., Ohiwa, N., Tateoka, M., Deocaris, C., Fujikawa, T. & Soya, H. (2007). Inhibitory effects of an orexin-2 receptor antagonist on orexin-A stress induced ACTH responses in conscious rats. *Neuroscience Research, 57,* 462-466.

Chiba, S., Numakawa, T., Ninomiya, M., Richards, M. C., Wakabayashi, C., & Kunugi, H. (2012). Chronic restraint stress causes anxiety- and depression-like behaviors, down regulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Progress in neuro-psychopharmacology & biological psychiatry*, *39*(1), 112-119. doi:10.1016/j.pnpbp.2012.05.018

De Lecea, L., Kilduff, T., Peyron, C., Gaos, X., FOye, P., Danielson, P., Fukuhara, C., Battenberg, E., Gautvik, V., Bartlett, F., Frankel, W., van den Pol, A., Bloom, F., Gautvik, K. & Sutcliffe, J. (1998). The hypocretins: Hypothalamus-specific peptides with neuroexcitatory activity. *Proceedings of the National Academy of Sciences, 95,* 322-327.

De Paula, H., Gouveia, A., de Almeida, M., & Hoshino, K. (2005). Anxiety levels and wild running susceptibility in rats: Assessment with elevated plus maze test and

predator odor exposure. *Behavioural Processes*, *68*(2), 135-144. doi:10.1016/j.beproc.2004.12.003

Del Cid-Pellitero, E. & Garzon, M. (2011). Hypocretin1/orexinA axon targeting of laterodorsal tegmental nucleus neurons projecting to the rat medial prefrontal cortex. *Cerebral cortex,* 1-12. doi: 10.1093/cercor/bhr070

DiMicco, J., Samuels, B., Zaretskaia, M., & Zaretsky, D. (2002). The dorsomedial hypothalamus and the response to stress: Part renaissance, part revolution. *Pharmacology, Biochemistry, and Behavior*, *71*(3), 469-80.

Ennaceur, A,, Michalikova, S., & Chazot, P. (2006). Models of anxiety: Responses of rats to novelty in an open space and an enclosed space. *Behavioural Brain Research*, *171*(1), 26-49. doi:10.1016/j.bbr.2006.03.016

Erami, E., Azhdari-Zarmehri, H., Rahmani, A., Ghasemi-Dashkhasan, E., Semnanian, S., & Haghparast, A. (2012). Blockade of orexin receptor 1 attenuates the development of morphine tolerance and physical dependence in rats. *Pharmacology, Biochemistry, and Behavior*, *103*(2), 212-9. Elsevier B.V. doi:10.1016/j.pbb.2012.08.010

Estabrooke, I., McCarthy, M., Ko, E., Chou, T., Chemelli, R., Yanagisawa, M., Saper, C. & Scammell, T. (2001). Fos expression in orexin neurons varies with behavioural state. *The Journal of Neuroscience*, *21*(5), 1656-1662.

Fernandes, C., González, M., Wilson, C, & File, S. (1999). Factor analysis shows that female rat behaviour is characterized primarily by activity, male rats are driven by sex and anxiety. *Pharmacology, Biochemistry, and Behavior*, *64*(4), 731-8.

Freeman, D., & Freeman, J. (2012). Anxiety: A very short introduction. Great Britain: Oxford University Press.

Furlong, T., Vianna, D., Liu, L. & Carrive, P. (2009). Hypocretin/orexin contributes to the expression of some but not all forms of stress and arousal. *European Journal of Neuroscience, 30,* 1603-1614.

Geerling, J., Mettenleiter, T. & Loewy, A. (2003). Orexins neurons project to diverse sympathetic outflow systems. *Neuroscience, 122,* 541-550.

Gentsch, C., Lichtsteiner, M., & Feer, H. (1981). Locomotor activity, defecation score and corticosterone levels during an open field exposure: A comparison among individually and group-housed rats, and genetically selected rat lines. *Physiology & Behaviour, 27*(1), 183-6.

Goldstein, L., Rasmusson, A., Bunney, B., & Roth, R. (1996). Role of the amygdala in the coordination of behavioral, neuroendocrine, and prefrontal cortical monoamine

responses to psychological stress in the rat. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *16*(15), 4787-98.

Gozzi, A., Turrini, G., Picoli, L., Massagrande, M., Amantini, D.,Antolini, M., Martinelli, P., Cesari, N., Montanari, D., Tessari, M., Corsi, M. & Bifone, A. (2011). Functional magnetic resonance imaging reveals different neural substrates for the effects of orexin-1 and orexin-2 receptor antagonists. *PLoS One*, *6*(1), 1-12. doi: 10.1371/journal.pone.0016406

Graeff, F, Netto, C., & Zangrossi, H. (1998). The elevated T-maze as an experimental model of anxiety. *Neuroscience and Biobehavioral Reviews*, *23*(2), 237-46.

Graeff, Frederico G, & Del-Ben, C. M. (2008). Neurobiology of panic disorder: From animal models to brain neuroimaging. *Neuroscience and Biobehavioral Reviews, 32*(7), 1326-35. doi:10.1016/j.neubiorev.2008.05.017

Greenberg P, Sisitsky T, Kessler R, Finkelstein S, Berndt E, Davidson J, Ballenger J, Fyer A. The economic burden of anxiety disorders in the 1990s. *Journal of Clinical Psychiatry* 1999;60(7):427–35.

Gulia, K., Mallick, H., & Kumar, V. (2003). Orexin A (hypocretin-1) application at the medial preoptic area potentiates male sexual behaviour in rats. *Neuroscience, 116,* 921-923. doi: 10.1016/S0306-4522(02)00877-1

Health Canada. (2009). Mental health: Anxiety disorders. Retrieved May 6, 2013, from http://www.hc-sc.gc.ca/hl-vs/alt_formats/pacrb-dgapcr/pdf/iyh-vsv/diseases-maladies/anxiety-anxieux-eng.pdf.

Henny, P. & Jones, B. (2006). Innervation of orexin/hypocretin neurons by GABAergic, glutamatergic or cholinergic basal forebrain terminals evidenced by immunostaining for presynaptic vesicular transporter and postsynaptic scaffolding proteins. *Journal of Comparative Neurology, 499*(4), 645-661

Hruba, L., Schutova, B. & Slamberova, R. (2012). Sex differences in anxiety-like behaviour and locomotor activity following prenatal and postnatal methamphetamine exposure in adult rats. *Physiology & Behaviour, 105,* 364-370.

Ida, T., Nakahara, K., Murakami, T., Hanada, R., Nakazato, M. & Murakami, N. (2000). Possible involvement of orexin in the stress reaction in rats. *Biochemical and Biophysical Research Communications, 270,* 318-323. doi: 10.1006/bbrc.2000.2412

Johnson, P. L., & Shekhar, A. (2012). An animal model of panic vulnerability with chronic disinhibition of the dorsomedial/perifornical hypothalamus. *Physiology & Behavior*, *107*(5), 686-98. Elsevier Inc. doi:10.1016/j.physbeh.2012.03.016

Johnson, P. L., Truitt, W. a, Fitz, S. D., Lowry, C. a, & Shekhar, A. (2008). Neural pathways underlying lactate-induced panic. *Neuropsychopharmacology*, *33*(9), 2093-107. doi:10.1038/sj.npp.1301621

Julien, R., Advokat, C. & Comaty, J. (2008). A primer of drug action: A comprehensive guide to the actions, uses, and side effects of psychoactive drugs. New York: Worth Publishers.

Kalinichev, M., & Holtzman, S. (2003). Changes in urination / defecation , auditory startle response , and startle-induced ultrasonic vocalizations in rats undergoing morphine withdrawal : Similarities and differences between acute and chronic dependence. *Pharmacology*, *304*(2), 603-609. doi:10.1124/jpet.102.044206.activity

Kastin, A., & Akerstrom, V. (1999). Orexin A but not orexin B rapidly enters brain from blood by simple diffusion. *The Journal of Pharmacology and Experimental Therapeutics*, *289*(1), 219-23.

Kaya, A., Karakaş, A., & Coşkun, H. (2011). The effects of the time of the day and the pinealectomy on anxiety-like behaviour in male Wistar rats. *Biological Rhythm Research*, *42*(5), 367-383. doi:10.1080/09291016.2010.525380

Kerman, I. (2008). Organization of brain somatomotor-sympathetic circuits. *Experimental brain research (Experimentelle Hirnforschung)*, *187*(1), 1-16. doi:10.1007/s00221-008-1337-5

Kessler, B., Stanley, E., Frederick-Duus, D. & Fadel, J. (2011). Age-related loss of orexin/hypocretin neurons. *Neuroscience*, *178*, 82-88. doi: 10.1016/j.neuroscience.2011.01.031

Kincheski, G. C., & Carobrez, A. P. (2010). The dorsal periaqueductal gray modulates the increased fear-like behaviour exhibited by experienced rats in the elevated plus-maze. *Behavioural Brain Research*, *206*(1), 120-6. doi:10.1016/j.bbr.2009.09.001

Kinn Rød, A., Milde, A, Grønli, J., Jellestad, F., Sundberg, H., & Murison, R. (2012). Long-term effects of foot shock and social defeat on anxiety-like behaviours in rats: relationships to pre-stressor plasma corticosterone concentration. *Stress*, *15*(6), 658-70. doi:10.3109/10253890.2012.663836

Kinzig, K. P., & Hargrave, S. L. (2010). Adolescent activity-based anorexia increases anxiety-like behaviour in adulthood. *Physiology & Behavior*, *101*(2), 269-76. doi:10.1016/j.physbeh.2010.05.010

Kirchgessner, A. L. (2002). Orexins in the brain-gut axis. *Endocrine Reviews*, *23*(1), 1-15.

Krout, K., Mettenleiter, T., Karpitskiy, V., Nguyen, X. & Lowey, A. (2005). CNS neurons with links to both mood-related cortex and sympathetic nervous system. *Brain Research, 1050,* 199-202. doi: 10.1016/j.brainres.2005.04.090

Kochenborger, L., Zanatta, D., Berretta, L. M., Lopes, A. P. F., Wunderlich, B. L., Januário, A. C., Neto, J. M., et al. (2012). Modulation of fear/anxiety responses, but not food intake, following α-adrenoceptor agonist microinjections in the nucleus accumbens shell of free-feeding rats. *Neuropharmacology*, *62*(1), 427-35. doi:10.1016/j.neuropharm.2011.08.019

Kodadek, T. & Cai, D. (2010). Chemistry and biology of orexin signalling. *Molecular Biosystems, 6,* 1366-1375. doi: 10.1039/c003468a

Kohtz, A., Paris, J., & Frye, C. (2010). Low doses of cocaine decrease, and high doses increase, anxiety-like behaviour and brain progestogen levels among intact rats. *Hormones and Behavior*, *57*(4-5), 474-80. doi:10.1016/j.yhbeh.2010.02.005

Kolaj, M., Coderre, E. & Renaud, L. (2008). Orexin peptides enhance median preoptic nucleus neuronal excitability via postsynaptic membrane depolarization and enhancement of glutamatergic afferents. *Neuroscience, 155,* 1212-1220.

Kokras, N., Dalla, C., Sideris, A. C., Dendi, A., Mikail, H. G., Antoniou, K., & Papadopoulou-Daifoti, Z. (2012). Behavioral sexual dimorphism in models of anxiety and depression due to changes in HPA axis activity. *Neuropharmacology*, *62*(1), 436-45. doi:10.1016/j.neuropharm.2011.08.025

Korczynksi, W., Ceregrzyn, M., Matyjek, R., Kato, I., Kuwahara, A., Wolinski, J. & Zabielski, R. (2006). Central and local (enteric) actions of orexins. *Journal of Physiology and Pharmacology, 57,* 17-42.

Kotz, C. (2006). Integration of feeding and spontaneous physical activity: Role of orexin. *Physiology & Behaviour, 88,* 294-301. doi: 10.1016/j.physbeh.2006.05.031

Kuru, M., Ueta, Y., Serino, R., Nakazato, M., Yamamoto, Y., Shibuya, I. & Yamashita, H. (2000). Centrally administered orexin/hypocretin activates HPA axis in rats. *Neuroendocrinology, 11*(9), 1977-1980.

Levay, E. a, Govic, A., Penman, J., Paolini, A. G., & Kent, S. (2007). Effects of adult-onset calorie restriction on anxiety-like behaviour in rats. *Physiology & Behavior*, *92*(5), 889-96. doi:10.1016/j.physbeh.2007.06.018

Levay, E. a, Paolini, A. G., Govic, A., Hazi, A., Penman, J., & Kent, S. (2008). Anxiety-like behaviour in adult rats perinatally exposed to maternal calorie restriction. *Behavioural Brain Research*, *191*(2), 164-72. doi:10.1016/j.bbr.2008.03.021

Li, Y., Li, S., Wei, C., Wang, H., Sui, N. & Kirouac, G. (2010a). Orexins in the paraventricular nucleus of the thalamus mediate anxiety-like responses in rats. *Psychopharmacology, 212,* 251-265. doi: 10.1007/s00213-010-1948-y

Li, Y., Li, S., Wei, C., Wang, H., Sui, N. & Kirouac, G. (2010b). Changes in emotional behaviour produced by orexin microinjections in the paraventricular nucleus of the thalamus. *Pharmacology, Biochemistry and Behavior, 95,* 121-128. doi: 10.1016/j.pbb.2009.12.016

Li, Y., Wang, X., Zhai, H., Zheng, Y., Zhang, X., Kosten, T., & Lu, L. (2008). Effects of early postnatal sibling deprivation on anxiety and vulnerability to cocaine in offspring rats. *Psychopharmacology*, *199*(2), 245-53. doi:10.1007/s00213-008-1169-9

Liang, C., Luo, H., Liu, Y., Cao, J., & Xia, H. (2012). Plasma hormones facilitated the hyper motility of the colon in a chronic stress rat model. *PloS One*, *7*(2), e31774. doi:10.1371/journal.pone.0031774

Lim, L. W., Blokland, A., van Duinen, M., Visser-Vandewalle, V., Tan, S., Vlamings, R., Janssen, M., et al. (2011). Increased plasma corticosterone levels after periaqueductal gray stimulation-induced escape reaction or panic attacks in rats. *Behavioural Brain Research*, *218*(2), 301-7. doi:10.1016/j.bbr.2010.12.026

Litvin, Y., Blanchard, D. & Blanchard, R. (2007). Rat 22 kHz ultrasonic vocalizations as alarm cries. *Behavioural Brain Research, 182,* 166-172.

Lowry, C., Johnson, P., Hay-Schmidt, A., Mikkelsen, J., & Shekhar, A. (2005). Modulation of anxiety circuits by serotonergic systems. *Stress*, *8*(4), 233-46. doi:10.1080/10253890500492787

Lopez, M., Tena-Sempre, M. & Dieguez, C. (2010). Cross-talk between orexins (hypocretins) and the neuroendocrine axes (hypothalamic-pituitary axes). *Frontiers in Neuroendocrinology, 31,* 113-127.

López-Aumatell, R., Martínez-Membrives, E., Vicens-Costa, E., Cañete, T., Blázquez, G., Mont-Cardona, C., Johannesson, M., et al. (2011). Effects of environmental and physiological covariates on sex differences in unconditioned and conditioned anxiety and fear in a large sample of genetically heterogeneous (N/Nih-HS) rats. *Behavioral and Brain Functions*, *7*, 48. doi:10.1186/1744-9081-7-48

Mallo, T., Alttoa, A., Koiv, K., Tonissaar, M., Eller, M. & Harro, J. (2007). Rats with persistently low or high exploratory activity: Behaviour in tests of anxiety and depression, and extracellular levels of dopamine. *Behavioural Brain Research, 177,* 269-281. doi: 10.1016/j.bbr.11.022

Marcus, J., Aschkensai, C., Lee, C., Chemelli, R., Saper, C., Yanagisawa, M. & Elmquist, J. (2001). Differential expression of orexin receptors 1 and 2 in the rat brain. *The Journal of Comparative Neurology, 435*, 6-25.

Markia, B., Kovacs, Z. & Palkovits, M. (2008). Projections from the vestibular nuclei to the hypothalamic paraventricular nucleus: morphological evidence for the existence of a vestibular stress pathway in the rat brain. *Brain Structure and Function,213*, 239-245. doi: 10.1007/s00429-008-0172-6

Martin, G., Fabre, V., Siggins, G. & de Lecea, L. (2002). Interactions of the hypocretins with neurotransmitters in the nucleus accumbens. *Regulatory Peptides, 104*, 111-117.

Martinez, G., Smale, L. & Nunez, A. (2002). Diurnal and nocturnal rodents show rhythms in orexinergic neurons. *Brain Research, 955*, 1-7.

Masini, C., Garcia, R., Sasse, S., Nyhuis, T., Day, H., & Campeau, S. (2010). Accessory and main olfactory systems influences on predator odor-induced behavioral and endocrine stress responses in rats. *Behavioural Brain Research*, *207*(1), 70-7. doi:10.1016/j.bbr.2009.09.038

McAuley, J., Stewart, A., Webber, E. S., Cromwell, H. C., Servatius, R., & Pang, K. (2009). Wistar-Kyoto rats as an animal model of anxiety vulnerability: Support for a hyper vigilance hypothesis. *Behavioural Brain Research*, *204*(1), 162-8. doi:10.1016/j.bbr.2009.05.036

Meyza, K., Boguszewski, P., Nikolaev, E., & Zagrodzka, J. (2011). Age increases anxiety and reactivity of the fear/anxiety circuit in Lewis rats. *Behavioural Brain Research*, *225*(1), 192-200. Elsevier B.V. doi:10.1016/j.bbr.2011.07.011

Miller, M., Morrison, J., & McEwen, B. (2012). Basal anxiety-like behaviour predicts differences in dendritic morphology in the medial prefrontal cortex in two strains of rats. *Behavioural Brain Research*, *229*(1), 280-288.doi:10.1016/j.bbr.2012.01.029

Muigg, P., Hetzenauer, A., Hauer, G., Hauschild, M., Gaburro, S., Frank, E., Landgraf, R., et al. (2008). Impaired extinction of learned fear in rats selectively bred for high anxiety--evidence of altered neuronal processing in prefrontal-amygdala pathways. *The European Journal of Neuroscience*, *28*(11), 2299-309. doi:10.1111/j.1460-9568.2008.06511.x

Mällo, T., Alttoa, A., Kõiv, K., Tõnissaar, M., Eller, M., & Harro, J. (2007). Rats with persistently low or high exploratory activity: Behaviour in tests of anxiety and depression, and extracellular levels of dopamine. *Behavioural Brain Research*, *177*(2), 269-81. doi:10.1016/j.bbr.2006.11.022

Mileykovskiy, B., Kiyashchenko, L. & Siegel, J. (2002). Muscle tone facilitation and inhibition after orexin-A (Hypocretin-1) microinjections into the medial medulla. *Journal of Physiology,87*, 2480-2489. doi: 10.1152/jn.00810.2001

Mönnikes, H., Schmidt, B. G., Tebbe, J., Bauer, C., & Taché, Y. (1994). Microinfusion of corticotropin releasing factor into the locus coeruleus/subcoeruleus nuclei stimulates colonic motor function in rats. *Brain Research, 644*(1), 101-8.

Nollet, M., Gaillard, P., Minier, F., Tanti, A., Belzung, C. & Leman, S. (2011). Activation of orexins neurons in the dorsomedial/perifornical hypothalamus and antidepressant reversal in a rodent model of depression. *Neuropharmacology, 61,* 336-346. doi: 10.1016/j.neuropharm.2011.04.022

Nozu T, Kumei S, Takakusaki K, Ataka K, Fujimiya M, Okumura T. (2011). Central orexin-A increases colonic motility in conscious rats. *Neuroscience Letters, 498*(2)143-6. doi: 10.1016/j.neulet.2011.04.078.

Ohno, K., Hondo, M., & Sakurai, T. (2008). Cholinergic regulation of orexin/hypocretin neurons through M3 muscarinic receptor in mice. *Journal of Pharmacological Sciences, 106*(3), 485-491. doi:10.1254/jphs.FP0071986

O'Kelly, L. (1940).The validity of defecation as a measure of emotionality in the rat. , *Journal of General Psychology, 23*, 75.

Pare. W. (1964). Relationship of various behaviours in the open-field test of emotionality. *Psychological Reports, 14,* 19-22.

Pawlak, C., Karrenbauer, B., Schneider, P., & Ho, Y. (2012). The elevated plus-maze test: Differential psychopharmacology of anxiety-related behavior. *Emotion Review, 4*(1), 98-115. doi:10.1177/1754073911421374

Paxinos, G. & Watson, C. (1977). The rat brain in stereotaxic coordinates. Academic Press: San Diego, CA.

Paxinos, G. & Watson, C. (2005).The rat brain in stereotaxic coordinates, 5th ed. Electronic text. Amsterdam; Boston: Academic Press

Pelloux, Y., Costentin, J., & Duterte-Boucher, D. (2009). Anxiety increases the place conditioning induced by cocaine in rats. *Behavioural Brain Research, 197*(2), 311-6. doi:10.1016/j.bbr.2008.08.029

Pereira-da-Silva, M., Cabral-Filho, J., & de-Oliveira, L. (2009). Effect of early malnutrition and environmental stimulation in the performance of rats in the elevated plus maze. *Behavioural Brain Research, 205*(1), 286-9. doi:10.1016/j.bbr.2009.05.031

Plotsky, P., Thrivikraman, K., Nemeroff, C., Caldji, C., Sharma, S., & Meaney, M.(2005). Long-term consequences of neonatal rearing on central corticotropin-releasing factor systems in adult male rat offspring. *Neuropsychopharmacology, 30*(12), 2192-204. doi:10.1038/sj.npp.1300769

Pradhan, N., & Arunasmitha, S. (1991). Correlations of motility, defecatory behaviour and striatal dopaminergic activity in rats. *Physiology & behaviour*, *50*(1), 135-8.

Prut, L., & Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: A review. *European Journal of Pharmacology*, *463*(1-3), 3-33. doi:10.1016/S0014-2999(03)01272-X

Ramos, A., Pereira, E., Martins, G., Wehrmeister. T. & Izídio, G. (2008). *Behavioural Brain Research,*193*(2),* 277-88. doi: 10.1016/j.bbr.2008.06.007

Reports, P., & Press, S. U. (1964). Relationship of various behaviors in the open-field test. *Public Health,* 14 (1), 19-22.

Rod, Milde, Gronli, Jellestad, Sundberg & Murison. (2012). *Stress,15*(6), 658-70. doi: 10.3109/10253890.2012.663836.

Roy, V, Chapillon, P., Jeljeli, M., Caston, J., & Belzung, C. (2009). Free versus forced exposure to an elevated plus-maze: Evidence for new behavioral interpretations during test and retest. *Psychopharmacology*, *203*(1), 131-41. doi:10.1007/s00213-008-1378-2

Russell, P. (1973). Open-field defecation in rats: Relationships with body weight and basal defecation level. *British Journal of Psychology, 64*(1), 109-114.

Rusyniak, D., Zaretsky, D., Zaretskaia, M., Durant, P. & DiMicco, J. (2012). *Physiology & Behaviour,* 1-8. doi: 10.1016/j.physbeh.2012.02.010

Sajdyk, T., Johnson, P., Fitz, S., & Shekhar, a. (2008). Chronic inhibition of GABA synthesis in the bed nucleus of the stria terminalis elicits anxiety-like behaviour. *Journal of Psychopharmacology*, *22*(6), 633-41. doi:10.1177/0269881107082902

Sakamoto, F., Yamada, S. & Ueta, Y. (2004). Centrally administered orexin-A activates corticotrophin-releasing factor-containing neurons in the hypothalamic paraventricular nucleus and central amygdaloid nucleus of rats: Possible involvement of central orexins on stress-activated central CRF neurons. *Regulatory Peptides, 118,* 183-191. doi: 10.1016/j.regpep.2003.12.014

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R., Tanaka, H., Williams, S., Richardson, J., Kozlowski, G., Wilson, S., Arch, J., Buckingham, R., Haynes, A., Carr, A., Annan, R., McNulty, D., Liu, W., Terrett, J., Elshourbagy, N., Bergsma, D. & Yanagisawa, M. (1998). Orexins and orexin receptors: A family of hypothalamic

neuropeptides and g protein-coupled receptors that regulate feeding behaviour. *Cell, 92,* 573-585

Sakurai, T. (2006). Roles of orexin and orexin receptors in central regulation of feeding behaviour and energy homeostasis. *CNS & Neurological Disorders-Drug Targets, 5,* 313-325.

Sakurai, T. & Meida, M. (2011). Connectonomics of orexin-producing neurons: Interface of the systems of emotion, energy homeostasis and arousal. *Trends in Pharmacological Science, 32*(8), 451- 462. doi: 10.1016/j.tips.2011.03.007

Salamon, R., Ripley, B., Kennedy, J., Johnson, B., Schmidt, D., Zeitzer, J., Nishino, S. & Mignot, E. (2003). Diurnal variation of cerebrospinal fluid hypocretin-1 (orexin-A) levels in control and depressed subjects. *Biological Psychiatry, 54,* 96-104. doi:10.1016/S0006-3223(03)01740-7

Salchner, P., Lubec, G., & Singewald, N. (2004). Decreased social interaction in aged rats may not reflect changes in anxiety-related behaviour. *Behavioural Brain Research*, *151*(1-2), 1-8. doi:10.1016/j.bbr.2003.07.002

Schroeder, M., & Weller, A. (2010). Anxiety-like behaviour and locomotion in CCK1 knockout rats as a function of strain, sex and early maternal environment. *Behavioural Brain Research*, *211*(2), 198-207. doi:10.1016/j.bbr.2010.03.038

Shekhar, A, McCann, U. D., Meaney, M. J., Blanchard, D. C., Davis, M., Frey, K. a, Liberzon, I., et al. (2001). Summary of a National Institute of Mental Health workshop: Developing animal models of anxiety disorders. *Psychopharmacology*, *157*(4), 327-39. doi:10.1007/s002130100859

Shekhar, A., Sajdyk, T., Gehlert, D. & Rainnie, D. (2003). The amygdala, panic disorder, and cardiovascular responses. *Annals of the New York Academy of the Sciences, 985,* 308-325.

Slattery, D., Uschold, N., Magoni, M., Bär, J., Popoli, M., Neumann, I. D., & Reber, S. O. (2012). Behavioural consequences of two chronic psychosocial stress paradigms: Anxiety without depression. *Psychoneuroendocrinology*, *37*(5), 702-14. doi:10.1016/j.psyneuen.2011.09.002

Smoller, J. W., Block, S. R., & Young, M. M. (2009). Genetics of anxiety disorders: The complex road from DSM to DNA. *Depression and Anxiety*, *26*(11), 965-75. doi:10.1002/da.20623

Strawn, J., Pyne-Geithman, G., Ekhator, N., Horn, P., Uhde, T., Shutter, L., Baker, D. & Geracioti Jr, T. (2010). Low cerebrospinal fluid and plasma orexin-A (hypocretin-1) concentrations in combat-related posttraumatic stress disorder. *Psychoneuroendocrinology, 35,* 1001-1007. doi: 10.1016/j.psycneuren.2010.01.001

Suzuki, M., Beuckmann, C., Shikata, K., Ogura, H. & Sawai, T. (2005). Orexin-A (hypocretin-1) is possibly involved in generation of anxiety-like behaviour. *Brain Research, 1044,* 116-121. doi: 10.1016/j.brainres.2005.03.002

Takakusaki, K., Takahashi,K., Saitoh, K., Harada, H., Okumura,T., Kayama, Y. & Koyama, Y. 2005). Orexinergic projections to the cat midbrain mediate alternation of emotional behavioural states from locomotion to cataplexy. *Journal of Physiology, 568.3,* 1003-1020. doi: 10.1113/jphysiol.2005.085829

Thorpe, A. & Kotz, C. (2005). Orexin A in the nucleus accumbens stimulates feeding and locomotor activity. *Brain Research, 1050,* 156-162. doi: 10.1016/j.brainres.2005.05.045

Trivedi, P., Yu, H., MacNeil, D. J., Van der Ploeg, L. H., & Guan, X. M. (1998). Distribution of orexin receptor mRNA in the rat brain. *FEBS Letters*, *438*(1-2), 71-5.

Tsujino, N., & Sakurai, T. (2013). Role of orexin in modulating arousal, feeding, and motivation. *Frontiers in Behavioral Neuroscience*, *7*(April), 28. doi:10.3389/fnbeh.2013.00028

Tyrka, A., Mello, A., Mello, M., Gagne, G., Grover, K., Anderson, G., Price, L., et al. (2006). Temperament and hypothalamic-pituitary-adrenal axis function in healthy adults. *Psychoneuroendocrinology*, *31*(9), 1036-45. doi:10.1016/j.psyneuen.2006.06.004

Viau, V., Sharma, S., & Meaney, M. (1996). Changes in plasma adrenocorticotropin, corticosterone, corticosteroid-binding globulin, and hippocampal glucocorticoid receptor occupancy/translocation in rat pups in response to stress. *Journal of Neuroendocrinology*, *8*(1), 1-8

Williams, D. & Russell, P. (1972). Open-field behaviour in rats: effects of handling, sex and repeated testing. *British Journal of Psychology*, *63(*4), 593- 596

Wilcock, J., & Broadhurst, P.. (1967). Strain differences in emotionality: Open-field and conditioned avoidance behaviour in the rat. *Journal of Comparative and Physiological Psychology*, *63*(2), 335-8.

Wilson, C., Vazdarjanova, A., & Terry, A. (2013). Exposure to variable prenatal stress in rats: Effects on anxiety-related behaviors, innate and contextual fear, and fear extinction. *Behavioural Brain Research, 238,* 279-88. doi:10.1016/j.bbr.2012.10.003

Yamanaka, A., Muraki, Y., Tsujino, N., Goto, K. & Sakurai, T. (2003). Regulation of orexin neurons by the monoaminergic and cholinergic systems. *Biochemical and Biophysical Research Communications, 303,* 120-129. doi: 10.1016/S0006-291X(03)00299-7

Yoshida, Y., Fujiki, N., Nakajima, T., Ripley, B., Matsumara, H., Yoneda, H., Mignot, E. & Nishino, S. (2001). Fluctuations of extracellular hypocretin-1 (orexin-A) levels in the rat in relation to the light-dark cycle and sleep-wake activities. *European Journal of Neuroscience*, *14,* 1075-1081.

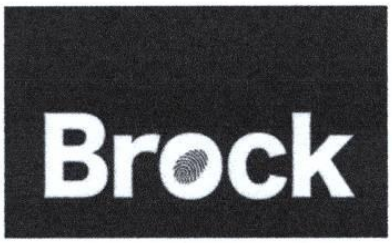
Yoshida, K., McCormick, S., Espana, R., Crocker, A. & Scammell, T. (2006). Afferents to the orexin neurons of the rat brain. *The Journal of Comparative Neurology*, *494,* 845-861. doi: 10.1002/cne/20859

Zhang, W., Zhang, N., Sakurai, T., & Kuwaki, T. (2009). Orexin neurons in the hypothalamus mediate cardiorespiratory responses induced by disinhibition of the amygdala and bed nucleus of the stria terminalis. *Brain Research*, *1262,* 25-37. doi:10.1016/j.brainres.2009.01.022

Zhang, J., Li, B., Yu, L., He, Y., Li, H., Zhu, J. & Wang, J. (2011). A role for orexin in central vestibular motor control. *Neuron, 69,* 793-804. doi: 10.1016/j.neuron.2011.01.026

Zhu, L., Onaka, T., Sakurai, T. & Yada, T. (2002). Activation of orexin neurons after noxious but not conditioned fear stimuli in rats. *Neuroendocrinology, 13*(10), 1351-1353.

Appendix



Animal Care and Use Committee (ACUC)
Chair –Fiona Hunter, PhD 905.688.5550 ext 3394
ACUC Veterinarian – Alistair Ker, DVM. 905.227.7644
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: April 15, 2013

Dear Dr. Brudzynski

Your "Animal Use Project Proposal (AUPP)" entitled:

Befriending the laboratory rat

has been approved by the Animal Care and Use Committee. This approval expires in one year on the last day of the month. The number for this project is **AUPP # 13 - 04 - 02.**
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 10 long evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Fiona Hunter, Chair of ACUC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

Rat Weights and Injections Administered

			I.C INJECTION CONDITION					
RAT ID	PRESURGICAL WEIGHT (GRAMS)	DECEASED	REMOVED FROM STUDY	LOCALIZATION	ORX-CARB	SAL-CARB	SAL-SAL	ORX-SAL
RMA1	306			OUT	☑	☑	☑	☑
RMA2	362			AHA	☑	☑7	☑	☑
RMA3	319			OUT	X	X	☑	☑
RMA4	343			OUT	X	X	☑	☑
RMA5	342			OUT	☑	☑	☑	☑
RMA6	437		YES		X	☑	☑	X
RMA7	471			OUT	X	☑	☑	X
RMA8	403			MPOA	☑	☑	☑	X
RMA9	397			AHA	X	☑	☑	☑
RMA10	413			MPOA	☑	☑	☑	☑
RMA11	430			MPOA	☑	☑	☑	X
RMA12	465			AHA	☑	☑	☑	☑
RMA13	315	YES			X	X	X	X
RMA14	340			AHA	X	☑	☑	X
RMA15	327			OUT	☑	☑	X	X
RMA16	320	YES		OUT	X	X	X	X
RMA17	320			OUT	X	☑	X	X
RMA18	331			OUT	☑	☑	☑	X
RMA19	370			OUT	X	☑	X	X
RMA20	395	YES		N/A	x	X	X	X
RMA21	433			N/A	☑	X	☑	X
RMA22				N/A	x	X	X	X
RMA23	430			AHA	☑	☑	X	X
RMA24	441			OUT	x	☑	☑	X
RMA25	267			N/A	x	☑	X	X
RMA26	305			N/A	x	☑	X	X
RMA27	277	YES			x	X	X	X
RMA28	275			AHA	x	☑	☑	X
RMA29	306	YES		N/A	x	X	X	X
RMA30	291			N/A	☑	☑	☑	X
RMA31	310			N/A	x	☑	X	X
RMA32	319	YES		N/A	x	X	X	X
RMA33	323	YES		N/A	x	X	X	X
RMA34	305			N/A	x	☑	X	X

RMA35	317	YES		N/A	**x**	**X**	**X**	**X**
RMA36	379			OUT	**x**	☑	☑	**X**
RMA37	337			OUT	**x**	☑	☑	**X**
RMA38	314			MPOA	**x**	☑	☑	**X**
RMA39	344			OUT	**x**	☑	☑	**X**
RMA40	427		YES	N/A	**x**	☑	**X**	**X**
RMA41	416			MPOA	**x**	☑	☑	**X**
RMA42	340			N/A	**x**	☑	**X**	**X**
RMA43	386			OUT	**x**	☑	☑	**X**
RMA44	367			N/A	**x**	☑	☑	**X**
RMA45	418			MPOA	**x**	☑	☑	**X**
RMA46	384			MPOA	**x**	☑	☑	**x**